AUTOIMMUNITY

Transglutaminase 4 as a prostate autoantigen in male subfertility

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Autoimmune polyendocrine syndrome type 1 (APS1), a monogenic disorder caused by *AIRE* gene mutations, features multiple autoimmune disease components. Infertility is common in both males and females with APS1. Although female infertility can be explained by autoimmune ovarian failure, the mechanisms underlying male infertility have remained poorly understood. We performed a proteome-wide autoantibody screen in APS1 patient sera to assess the autoimmune response against the male reproductive organs. By screening human protein arrays with male and female patient sera and by selecting for gender-imbalanced autoantibody signals, we identified transglutaminase 4 (TGM4) as a male-specific autoantigen. Notably, TGM4 is a prostatic secretory molecule with critical role in male reproduction. TGM4 autoantibodies were detected in most of the adult male APS1 patients but were absent in all the young males. Consecutive serum samples further revealed that TGM4 autoantibodies first presented during pubertal age and subsequent to prostate maturation. We assessed the animal model for APS1, the *Aire*-deficient mouse, and found spontaneous development of TGM4 autoantibodies specifically in males. *Aire*-deficient mice failed to present TGM4 in the thymus, consistent with a defect in central tolerance for TGM4. In the mouse, we further link TGM4 immunity with a destructive prostatitis and compromised secretion of TGM4. Collectively, our findings in APS1 patients and *Aire*-deficient mice reveal prostate autoimmunity as a major manifestation of APS1 with potential role in male subfertility.

INTRODUCTION

Male reproductive dysfunction is found in every 13th couple attempting to conceive (*1*–*3*). Infertility in males may result from diseases affecting the gonads or from dysfunctions at the pretesticular or posttesticular level (*1*–*3*). The male accessory gland secretion forms the major bulk of the ejaculate and contributes with factors critical for sperm survival and maturation (*4*). The prostate is the largest of the male accessory glands and is located at the base of the urine bladder, surrounding the urethra (*4*). During puberty, the prostate matures into an active secretory organ and initiates the expression of a repertoire of secretory proteins with specialized functions in the semen. Prostatic secretion also contains high amounts of zinc, calcium, phosphate, and citric acid, which are important in controlling semen pH, chromatin stabilization, and spermatogenesis (*4*). Prostatitis may impair prostate functions and, through multiple and incompletely defined mechanisms, affect fertility (*4*–*8*).

Autoimmune polyendocrine syndrome type 1 (APS1) (Mendelian Inheritance in Man number 240300) is a monogenic disorder that features multiple disease manifestations in endocrine and nonendocrine organs (*9*). The disease is caused by mutations in the autoimmune regulator (*AIRE*) gene on chromosome 21 and is recessively inherited. APS1 and its animal model have been instrumental for the elucidation of molecular mechanisms underlying central immune tolerance and its role in autoimmunity (*10*, *11*). *Aire* promotes the expression of a wide array of tissue-specific antigens in medullary thymic epithelial cells (mTECs), and this presentation of self-antigens is crucial for the negative selection of autoreactive T cells. Most APS1 patients develop a triad of chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency, which are considered hallmark components of the disease (*9*). Many patients also acquire additional autoimmune manifestations such as type 1 diabetes mellitus, pernicious anemia, malabsorption, vitiligo, and alopecia (*9*). The autoimmune disease repertoire of APS1 has been extended successively, with further characterization of clinical features and autoantibody responses. For example,

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the identification of a bronchiolar autoantigen enabled definition of lung disease as a component of APS1 (*12*).

Infertility is common in both females and males with APS1, and only a few patients in stable relationships give birth to or father children (*9*). Autoimmune ovarian failure is found in most adult female APS1 patients (*9*) and is believed to be the dominating etiology behind female infertility. However, in male APS1 patients gonadal failure is rare (*9*), and also male adults without gonadal failure often remain childless (*13*). This has raised the question whether other disease mechanisms, yet to be identified, contribute to subfertility in male APS1 patients. The animal model of APS1, the *Aire*-deficient mouse, reproduces multiorgan autoimmunity and male subfertility (*10*, *14*, *15*). *Aire*-deficient males and females show severely reduced fertility in intercrosses (*14*, *15*), where the fertility defect most often relies in the male (*15*, *16*). Male subfertility is further apparent in breedings between *Aire*-deficient males and wild-type females (*14*, *16*). The cause for male subfertility in *Aire*-deficient mice has not been established and does not seem to be explained by gonadal autoimmunity. Whereas the testis anatomy and histology appear normal (*14*, *15*), the prostate gland is a major target of autoimmune destruction (*17*–*19*).

We used a proteomic approach to assess the autoimmune response against male reproductive organs in APS1, in an effort to reveal previously unknown etiologies for male subfertility. By screening human protein arrays with sera from male and female APS1 patients and by selecting for male-specific autoantibody signals, we identified transglutaminase 4 (TGM4) as a major prostate autoantigen. In consecutive serum samples, we show that TGM4 autoantibodies first present during pubertal age and subsequent to prostate maturation. Moving to the *Aire*-deficient mouse model, we link TGM4 immunity with a tissue-destructive prostatitis and failing secretion of TGM4. Collectively, our findings indicate that prostate autoimmunity is a major manifestation of APS1 with potential implications on male fertility.

RESULTS

Proteome-wide screen identifies TGM4 as a male-specific autoantigen

We used human protein arrays to perform a proteome-wide screen for male-specific autoantigens in APS1. Protein arrays containing more than 9000 human proteins were probed with sera from 27 male and 24 female APS1 patients and 21 healthy control subjects, and immunoglobulin G (IgG) autoantibodies were detected. We used a two-step selection process to identify gender-imbalanced autoantigens: first by selecting for patient specificity, and second by assessing the top targets for gender specificity (Fig. 1A). As a first filtration, we removed all targets where no patient had an intensity >5000 or where the negative sample (array probed without serum sample) had an intensity >2000, which left us with a selection of 429 targets. Autoantibody signal values were log-transformed, and *t* test was used to identify targets that differed between APS1 patients and healthy controls. The 50 targets most strongly associated with the APS1 patient group were selected and assessed for gender specificity. Cutoff values were introduced for each target at 3 SDs above the average of the healthy control group, and the frequency of positive individuals was compared between male and female APS1 patients by Fisher's exact test. At the 0.05 significance level and after the Bonferroni correction for multiple testing, only one significant target remained—TGM4 [$n = 27 + 24$; $P = 0.00017$ (not adjusted), Fisher's exact test]. TGM4 is a prostate-specific enzyme (*20*, *21*) with a central role in male reproductive physiology (*22*–*26*). Autoantibodies against TGM4 were detected in 14 male patients and 1 female patient and were absent in healthy subjects (Fig. 1B).

TGM4 autoantibodies are specific for APS1

To confirm TGM4 as a valid autoantigen, we used a radioligand binding assay (RLBA) to screen an extended cohort of 93 APS1 patients and more than 500 healthy and disease control subjects for TGM4 autoantibodies. Reinvestigation of the 51 APS1 patients confirmed TGM4 autoantibodies in all 14 male patients and in 1 female patient classified as reactive on the array. Two additional males were identified as TGM4-reactive in the RLBA, whereas the remaining 34 patients were confirmed negative (Fig. 2A, fig. S1, and table S1). A replication cohort including 19 male and 23 female APS1 patients was screened for TGM4 autoantibodies, where another 10 reactive male patients were identified (Fig. 2B and table S1). In total, we detected TGM4

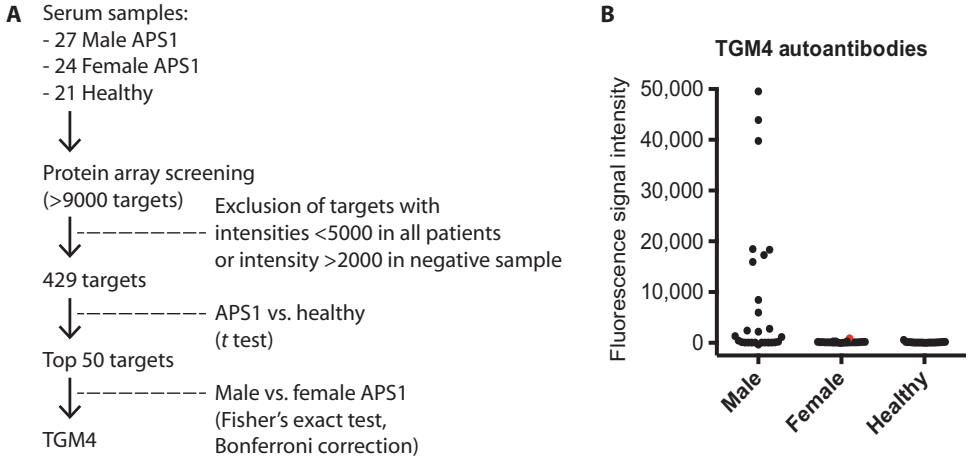

Fig. 1. Identification of TGM4 as a male-specific autoantigen. (**A**) Human protein arrays containing more than 9000 targets were screened with sera from 27 male APS1 patients, 24 female APS1 patients, and 21 healthy control subjects, and gender-imbalanced autoantigens were selected. After an initial filtering removing targets where no patient had an intensity > 5000 or where the negative sample had an intensity > 2000, 429 targets remained. A *t* test was used to identify targets that differed between APS1 patients and healthy controls, and the top 50 targets were then assessed for gender specificity by comparing the frequency of positive individuals between male and female APS1 patients using Fisher's exact test (cutoff = average of the healthy + 3 SD). At the 0.05 significance level and after the Bonferroni correction for multiple testing, only one significant target remained—TGM4 [n = 27 + 24; $P = 0.00017$ (not adjusted), Fisher's exact test)]. (**B**) TGM4 autoantibodies were detected in 14 male patients and 1 female patient (marked in red) and were absent in the healthy subjects (cutoff = average of the healthy + 3 SD). The *y* axis indicates the average fluorescence signal intensity for duplicate TGM4 protein spots on the array, after subtraction of the background signal.

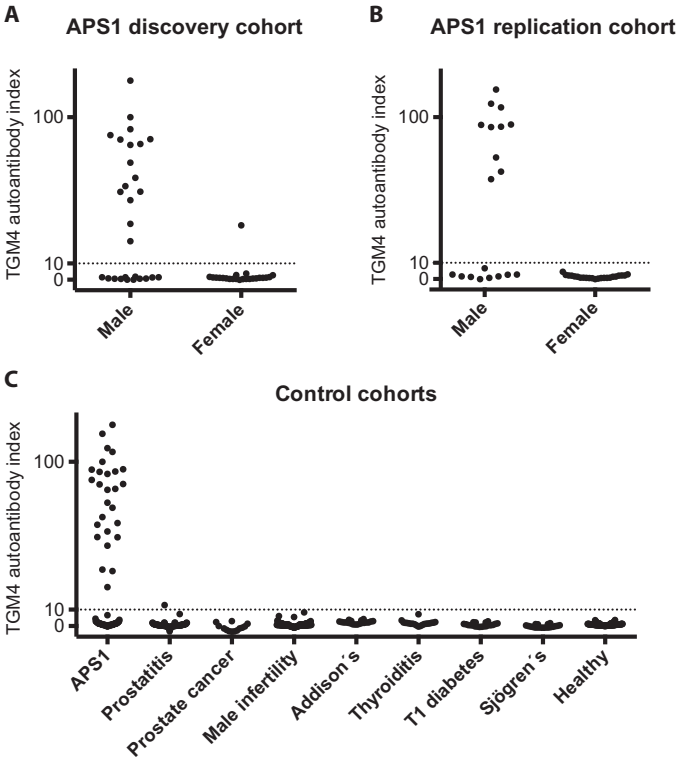

Fig. 2. TGM4 autoantibodies are highly specific for male APS1 patients. TGM4 autoantibodies were validated in an extended clinical material using an RLBA. (**A** and **B**) TGM4 autoantibodies were confirmed in a discovery cohort of 27 male and 24 female APS1 patients (**A**) and were also demonstrated in a replication cohort of 19 male and 23 female APS1 patients (**B**). In total, we detected TGM4 autoantibodies in 26 male APS1 patients (57%) and 1 female APS1 patient. (**C**) To determine the clinical specificity of TGM4 autoantibodies, we also investigated 98 patients with prostatitis, 14 patients with prostate cancer, 160 males with idiopathic infertility, a selection of autoimmune diseases (including 20 patients with each of Addison's disease, autoimmune thyroiditis, type 1 diabetes mellitus, and Sjögren's syndrome), and 135 healthy control subjects. TGM4 autoantibodies were detected just above cutoff in one patient with prostatitis and were absent in remaining controls, and thereby showed high specificity for male APS1 patients. The upper limit of the normal range was defined as an index value of 10.

autoantibodies in 26 of 46 (57%) male APS1 patients and in 1 of 47 female APS1 patients. To determine the clinical specificity of TGM4 autoantibodies, we also investigated 98 patients with prostatitis, 14 patients with prostate cancer, 160 males with idiopathic infertility, a selection of autoimmune diseases (n = 80), and 135 healthy subjects. One patient with prostatitis was found with TGM4 autoantibody level just above cutoff, whereas all infertile males, prostate cancer patients, autoimmune disease patients, and healthy subjects were negative for TGM4 autoantibodies (Fig. 2C). TGM4 autoantibodies thereby appeared to be highly specific for male APS1 patients.

Several members of the transglutaminase family have been identified as immune targets in distinct autoimmune disorders (*27*–*30*) (Fig. 3A). Tissue transglutaminase (TGM2) is the major autoantigen in celiac disease (*27*) and displays 33% protein sequence identity with TGM4. We assessed the target specificity of transglutaminase autoantibodies in APS1 as compared with celiac disease, and immunoprecipitated radiolabeled TGM4 and TGM2 protein with sera from the APS1 patients and a cohort of 50 patients with celiac disease. APS1 patients specifically reacted with TGM4, whereas patients with celiac disease only recognized TGM2 (Fig. 3B).

TGM4 is specifically expressed in prostate epithelial cells

Previous studies have established TGM4 as a prostate-specific marker (*20*, *21*). For validation, we investigated the expression of TGM4 in a broad selection of human tissues and assessed both the mRNA and protein levels. TGM4 mRNA was measured in 11 human tissue samples with digital droplet polymerase chain reaction (PCR) and was specifically detected in the prostate gland (Fig. 4A and fig. S2). A polyclonal antibody was used to characterize the distribution of TGM4 protein in a panel of more than 15 tissues, and antibody binding was detected by immunohistochemistry and by immuno-rolling circle amplification. Distinct staining was seen in the prostate epithelium, whereas remaining tissues demonstrated very faint or no staining (Fig. 4B, figs. S3 and S4). TGM4 expression was further restricted to specific regions within the prostate gland, consistent with previous reports (*31*) (Fig. 4C).

TGM4 autoantibodies present at puberty

TGM4 expression is androgen-driven (*21*, *32*), and studies of TGM4 in mouse and other prostate markers in humans suggest that TGM4 expression begins during early puberty (*33*–*35*). We therefore looked at the prevalence of TGM4 autoantibodies in male patients of pre-and postpubertal age, respectively. TGM4 autoantibodies were seen in 78% of males more than 30 years of age (n = 18) and in 52% of males 13 to 30 years of age (n = 23). However, the five patients younger than 13 years were all TGM4 autoantibody–negative (Fig. 5A). To exclude the effect of an unspecific age bias, we also investigated the prevalence by age of autoantibodies against established APS1 autoantigens, including 21-hydroxylase, glutamic acid decarboxylase 65 (GAD65), 17-hydroxylase, side-chain cleavage enzyme, tyrosine hydroxylase, tryptophan hydroxylase, aromatic L amino acid decarboxylase, and cytochrome P450 1A2 (CYP1A2), and all these autoantibodies were detected in patients belonging to the youngest age category (table S2). To further clarify the relation between TGM4 autoantibodies and puberty, we analyzed consecutive samples collected from TGM4-reactive patients and selected six males with available samples from young age. TGM4 autoantibodies were first detected between the ages of 12 and 16 years in the six patients, and reactivity was thereafter sustained in successive samples (Fig. 5B and table S3). None of the males had developed TGM4 autoantibodies before the age of an expected pubertal debut.

Aire-deficient mice develop TGM4 autoantibodies and prostatitis

The animal model of APS1, the *Aire* knockout mouse, spontaneously develops autoimmune manifestations in multiple organs (*10*, *14*) including the prostate gland (*17*–*19*). We investigated the spontaneous development of prostatitis in the *Aire*-deficient mouse model and assessed the involvement of TGM4. Serum autoantibodies against murine TGM4 were measured in 66 mice from the C57BI/6 genetic background, including 23 *Aire*-deficient males, 24 *Aire*-deficient females, 11 wild-type males, and 8 wild-type females. TGM4 autoantibodies were present in all male *Aire*-deficient mice but were absent in all female *Aire*-deficient mice and all wild-type mice

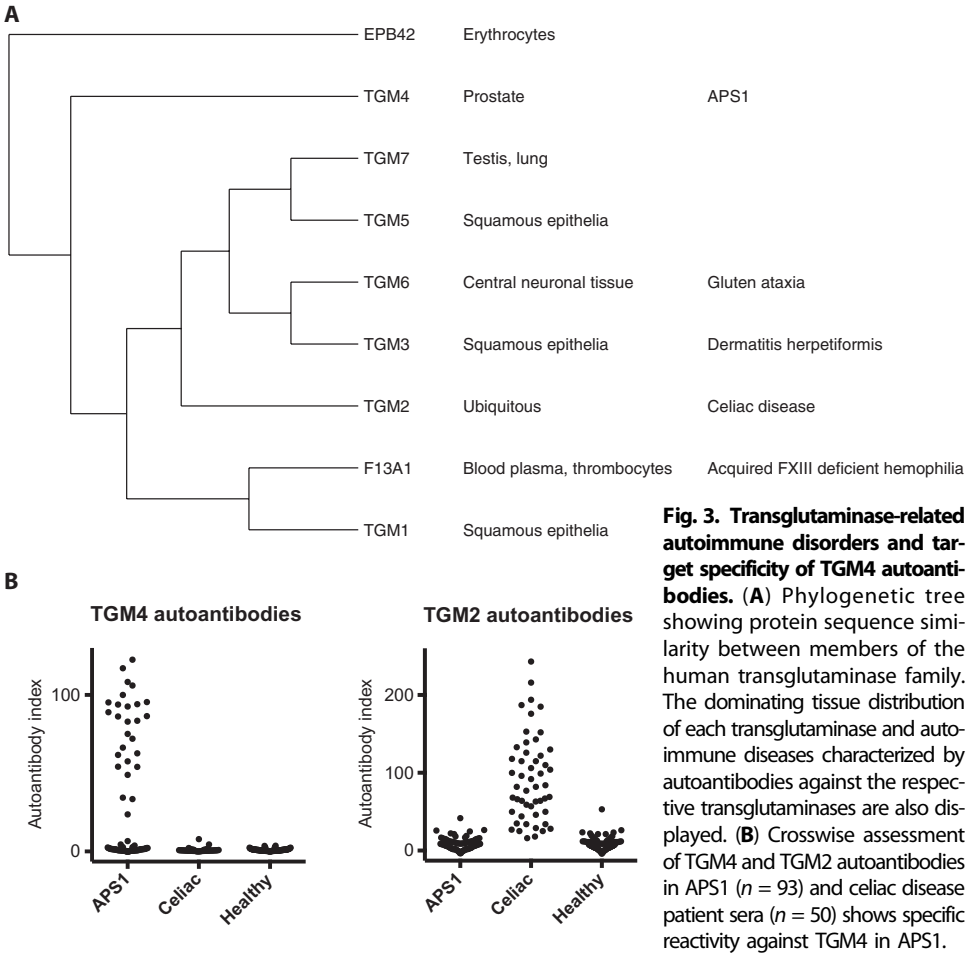

Fig. 3. Transglutaminase-related autoimmune disorders and target specificity of TGM4 autoantibodies. (**A**) Phylogenetic tree showing protein sequence similarity between members of the human transglutaminase family. The dominating tissue distribution of each transglutaminase and autoimmune diseases characterized by autoantibodies against the respective transglutaminases are also displayed. (**B**) Crosswise assessment of TGM4 and TGM2 autoantibodies in APS1 (n = 93) and celiac disease patient sera (n = 50) shows specific reactivity against TGM4 in APS1.

(Fig. 6A). To assess the broader relevance of TGM4 as a prostate autoantigen, we also investigated another model of spontaneous autoimmune prostatitis—the nonobese diabetic (NOD) mouse (*36*). *Aire*-deficient (n = 22) and wild-type mice (n = 30) of the NOD background were screened for TGM4 autoantibodies. As with the C57BI/6 strain, we detected TGM4 autoantibodies in all *Aire*-deficient mice of male sex (n = 7), whereas female *Aire*-deficient mice were negative (n = 15) (fig. S5). We also found male-specific TGM4 autoantibodies in wild-type mice of the NOD strain, detecting TGM4 autoantibodies at a high level in two of nine males (22%), whereas female mice were negative (n = 21) (fig. S6), indicating a more general role of TGM4 in prostatitis.

We next investigated the prostate histology in *Aire*-deficient mice with TGM4 autoantibodies as compared with wild-type mice (all from the NOD strain). The *Aire*-deficient mice displayed typical signs of an active prostatitis, with massive mononuclear cell infiltrates in the prostate interstitium and epithelium, whereas prostate histology in wild-type mice was normal (Fig. 6B). To characterize the prostate-infiltrating T cells, we assessed markers for T helper 1 (T_H1), T_H2, T_H17, and T regulatory (T_{reg}) cell subsets in prostate tissue from *Aire*-deficient and wild-type NOD mice. The T_H1-associated cytokine interferon-γ (IFNγ) showed high expression in the *Aire*-deficient mice compared to wild type. Foxp3 also appeared to be elevated in *Aire*-deficient mouse prostates, whereas T_H2-associated cytokines (*Il4* and *Il13*) and T_H17-associated molecules (*Rorc*, *Il17a*, and *Il17f*) did not show a significant increase in the *Aire*-deficient mice (Fig. 6C and table S4). Affected prostates thereby appeared to show an increase in T_H1 cells, which are critical for autoimmune pathology in *Aire* deficiency (*37*).

Aire drives thymic expression of TGM4

Aire promotes the expression of a repertoire of tissue-specific self-antigens in mTECs that is essential for negative T cell selection (*10*). We investigated the thymic expression of TGM4 to assess the role of central immune tolerance in TGM4 immunity. mTECs were isolated from wild-type and *Aire*-deficient mice from the C57BI/6 strain and were subjected to mRNA expression analyses by quantitative PCR (qPCR). As a comparison for TGM4, we also included *Ins2* and *Crp* to the analyses, the former being an established *Aire*-dependent gene and the latter being expressed in mTECs independently of *Aire*. TGM4 was detected in mTECs from wild-type mice but was absent in mTECs from *Aire*-deficient mice, similar to the results for *Ins2* and distinct from *Crp* (Fig. 7 and table S5). TGM4 thereby showed *Aire*-dependent thymic expression, consistent with a defect of central tolerance for TGM4 in *Aire*-deficient subjects.

Aire-deficient mice fail to secrete TGM4

Male *Aire*-deficient mice display reduced fertility of unknown cause (*14*–*16*). Studies in TGM4 knockout mice and in other experimental systems have shown that prostatic secretion of TGM4 is critical for male fertility (*22*–*26*). We therefore investigated whether prostatic TGM4 secretion was affected in *Aire*-deficient mice and could explain their compromised fertility. TGM4 mRNA levels were assessed in prostate tissues collected from *Aire*-deficient NOD mice with TGM4 autoantibodies and from wild-type NOD mice. Notably, prostates from the *Aire*-deficient mice showed absent levels of TGM4 (Fig. 8 and table S4). Prostate autoimmunity thereby appeared to have rendered the *Aire*-deficient mice with a complete deficiency of prostatic TGM4.

DISCUSSION

This work identifies prostate autoimmunity as a hitherto unrecognized manifestation of APS1 and TGM4 as a male-specific autoantigen. *Aire*-deficient mice with TGM4 autoantibodies displayed prostatitis and failing secretion of TGM4. Given the important role of the prostate gland (*4*) and TGM4 function (*22*–*26*) in male reproductive physiology, it is plausible that prostate autoimmunity contributes to subfertility in male APS1 patients.

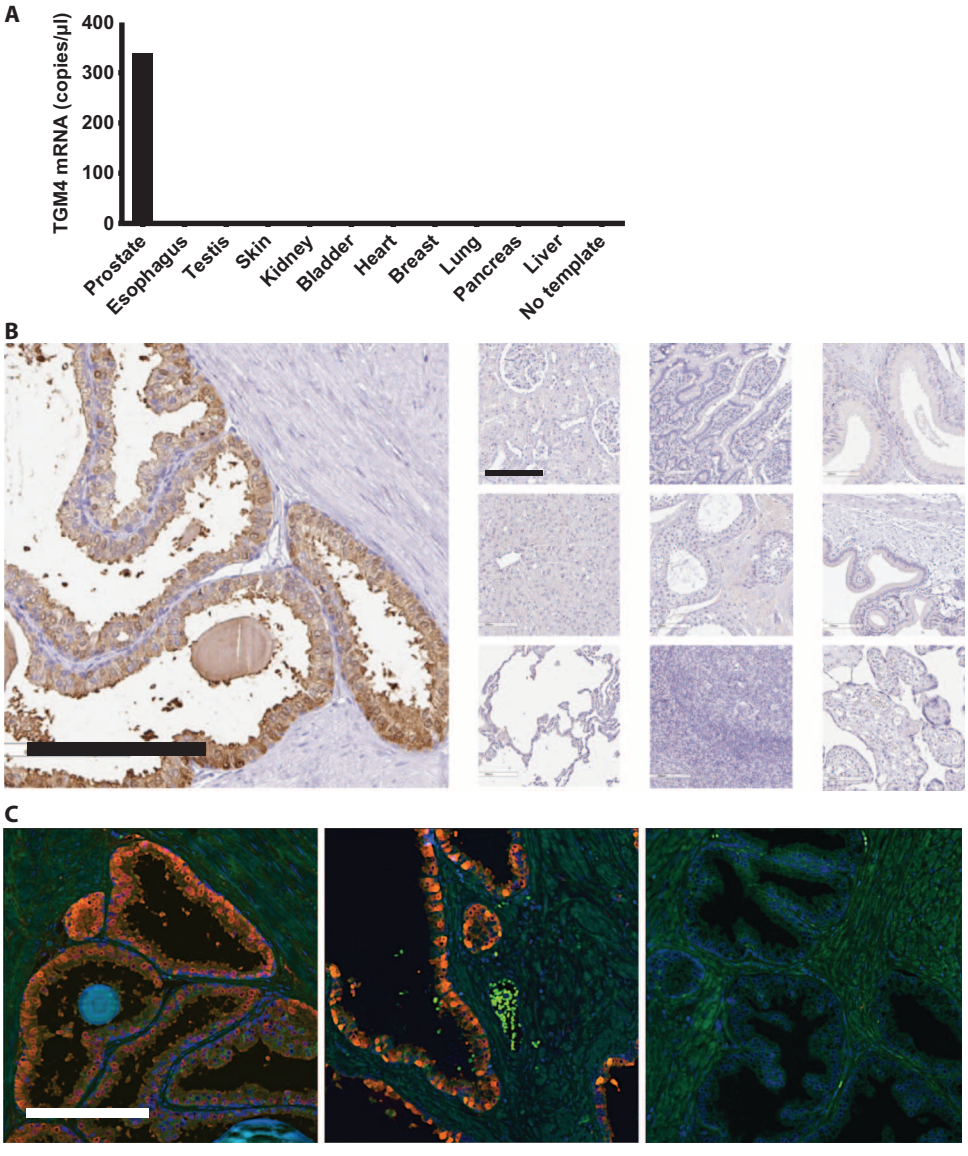

Fig. 4. TGM4 is specifically expressed in the prostate epithelium. The expression of TGM4 was investigated on mRNA level by digital droplet PCR and on protein level by immunohistochemistry and immuno-rolling circle amplification, and multiple human tissues were assessed. (**A**) TGM4 mRNA was specifically detected in prostate tissue. (**B**) Strong TGM4 staining was seen in the prostate epithelium, whereas remaining tissues showed very faint or no staining (from left to right: prostate, kidney, duodenum, epididymis, liver, testis, gallbladder, lung, tonsil, and placenta). (**C**) TGM4 expression was further restricted to certain regions within the prostate gland, here shown with immuno-rolling circle amplification and exemplified by a tissue section with marked TGM4 expression (left), a transition zone in which the prostate epithelium contains cells that are positive and cells that are negative for TGM4 (middle), and a region without TGM4 expression (right). Scale bars, 200 µm.

APS1 is a valuable model for tissue-specific autoimmune disease and has been instrumental for the understanding of autoimmune mechanisms in female infertility (*38*). Here, we searched for unrecognized etiologies of male subfertility and adopted a proteomics approach to assay the autoimmune response against male reproductive organs. By selecting for male-specific immune targets, we identified TGM4 as a major prostate autoantigen. TGM4 autoantibodies were confirmed by independent methods in an extended APS1 patient cohort, were detected in most male APS1 patients, and proved to be highly specific for APS1 in a broad selection of disease cohorts. In a time series of samples, we could show that TGM4 autoantibodies first presented during pubertal age, when the prostate matures into an active secretory organ and TGM4 expression commences. We then used the *Aire*-deficient mouse model to better understand the underlying mechanisms and consequences of TGM4 immunity. The *Aire*-deficient mouse displays autoimmune prostatitis (*17*–*19*) and male subfertility (*14*–*16*), and therefore represents an excellent model system for the translation of our findings in APS1. Sera from *Aire*-deficient mice have previously been shown to immunoprecipitate a band in prostate extracts with a size corresponding to TGM4 (*17*). Using an autoantibody assay for murine TGM4, we could demonstrate spontaneous formation of TGM4 autoantibodies specifically in male *Aire*-deficient mice, and thereby establish a link between TGM4 immunity and the development of an inflammatory and tissue-destructive prostatitis. We also found *Aire*-dependent thymic expression of TGM4 despite negative results from one previous study (*18*), revealing a defect in central tolerance for TGM4 in *Aire*-deficient subjects.

Fertility problems appear to be common in male APS1 patients (*9*, *13*, *39*) but have not been well characterized. Gonadal failure is a recognized cause of infertility in male APS1 patients but is only seen in a few patients (*9*). We here uncover prostate autoimmunity as a major manifestation of APS1, identifying a novel mechanism that could contribute to subfertility in male APS1 patients. TGM4 appears to have a key role in male reproductive physiology through different mechanisms. TGM4 is a major regulator of semen viscosity and promotes semen coagulation by cross-linking gel-forming proteins (*22*, *40*). The TGM4 knockout mouse displays severely reduced male fertility, as partially explained by insufficient ejaculate migration in the female reproductive tract and partially by an effect on postfertilization events such as implantation and gestation (*22*). Prostatic transglutaminase-mediated modifications of the sperm surface further appears to be important for sperm capacitation, whereby the male gamete acquires features of a fully differentiated fertile cell (*24*, *25*), and for protecting sperm from immunological attack in the female reproductive tract (*23*, *26*). *Aire*-deficient mice with TGM4 autoantibodies lacked production of TGM4, suggesting that TGM4-expressing prostatic ducts had been

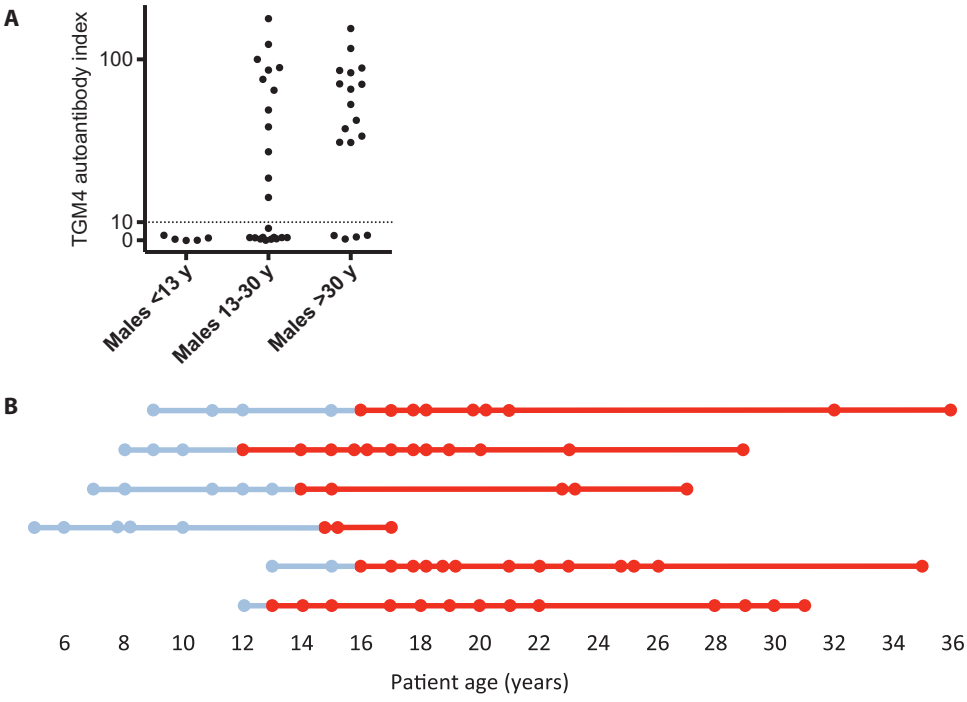

Fig. 5. TGM4 autoantibodies present at puberty. (**A**) TGM4 autoantibodies were detected in 78% of males more than 30 years of age (n = 18) and in 52% of males 13 to 30 years of age (n = 23), but were absent in the five patients younger than 13 years. (**B**) TGM4 autoantibodies were assessed in consecutive samples from six male APS1 patients and were first detected by the age of 12 to 16 years. Time points for blood sampling are indicated as dots along the patient age timeline, and TGM4 autoantibody positivity is marked in red. TGM4 autoantibodies were measured in serum using an RLBA, and the upper limit of the normal range was defined as an index value of 10.

destroyed by the autoimmune reaction. This failure to secrete prostatic TGM4 should be expected to have the same consequences on fertility as seen in the TGM4 knockout mouse. *Aire*-deficient male mice show reduced sperm quality and fertilization success despite apparently normal testes (*15*). Such effects on sperm quality and fertilization success could indeed be expected from a TGM4 deficiency given the role of TGM4 in sperm maturation (*24*, *25*). However, other mechanisms whereby prostate autoimmunity may affect male fertility must also be considered. For example, inflammatory mediators in prostatitis have been shown to exert toxic effect and oxidative stress on spermatozoa (*5*).

TGM4 is a member of the transglutaminase family that includes nine human genes (*41*, *42*). Transglutaminases catalyze a variety of post-translational protein modifications, but are most recognized as protein cross-linkers forming covalent bonds between lysine and glutamine residues (*41*). Several members of the transglutaminase family have been identified as major autoantigens in distinct autoimmune disorders. Celiac disease, as characterized by autoantibodies specific for TGM2 (*27*), may be complicated by manifestations in the skin and nervous system. Dermatitis herpetiformis is associated with autoantibodies against epidermal transglutaminase (TGM3) (*28*), whereas gluten-sensitive cerebellar ataxia has been linked to autoantibodies against neuronal transglutaminase 6 (TGM6) (*29*). Acquired factor XIII (FXIII) deficiency hemophilia is caused by inhibitory autoantibodies against coagulation FXIII (F13A1)—a transglutaminase that is needed for the cross-linking of fibrin in the last step of the coagulation cascade (*30*). The recognition of TGM4-specific autoantibodies in APS1 further adds to the range of transglutaminase autoantibody–related diseases and also raises the question if there are yet more transglutaminase family members to be implicated with autoimmune disease.

Autoantibodies in tissue-destructive autoimmune diseases typically target intracellular antigens, and as with autoantibodies against thyroid peroxidase in autoimmune thyroiditis or autoantibodies against 21-hydroxylase in Addison's disease, TGM4 autoantibodies are not expected to exert a direct pathogenic effect. More likely, these autoantibodies may represent a marker of tissue-specific autoimmunity that involves both humoral and cellular arms and where the latter is the effector of the destructive insult.

Another autoantigen that has previously been implicated in prostatitis in the *Aire*-deficient mouse is seminal vesicle protein 2 (SVS2), which is the mouse homolog of human semenogelin (*19*). Semenogelin is the major gel-forming protein in semen and an important substrate for TGM4 (*40*). Similar situations, where an enzyme and its substrate both become targeted by autoantibodies, have been described in other autoimmune disorders. Celiac disease is characterized by autoantibodies against TGM2 and its substrate, gliadin (*27*), and patients with rheumatoid arthritis display autoantibodies against citrullinated peptides and the enzyme catalyzing these modifications, peptidyl arginine deiminase 4 (PADI4) (*43*).

The prostate tissue morphology could not be assessed in the APS1 patients because such investigations could not be ethically motivated. Future studies investigating prostate tissue collected from male APS1 patients undergoing clinically motivated prostate biopsies will be important to evaluate the consequences of prostate autoimmunity. Because TGM4 expression is restricted to certain regions within the prostate gland, a tissue-destructive insult of the prostate would not be expected to affect the whole gland. Multiple prostate biopsies and anti-TGM4 staining would probably be required to detect a specific loss of TGM4-expressing prostatic ducts after long-standing disease.

Only one female patient in the APS1 cohort was found to harbor TGM4 autoantibodies. Review of the medical record of this 42-year-old patient revealed that she was prescribed fluoxymesterone, a steroid with strong androgenic properties, at a daily dose of 2.5 to 5 mg from the age of 20 to 25 years. Studies in experimental animals and in humans have shown that administration of androgens in females induces the production of prostate secretory proteins in Skene's glands, also known as the female prostate (*44*, *45*). Androgen administration in this female APS1 patient could therefore have induced the expression of TGM4 in Skene's glands, mimicking the situation in postpubertal males.

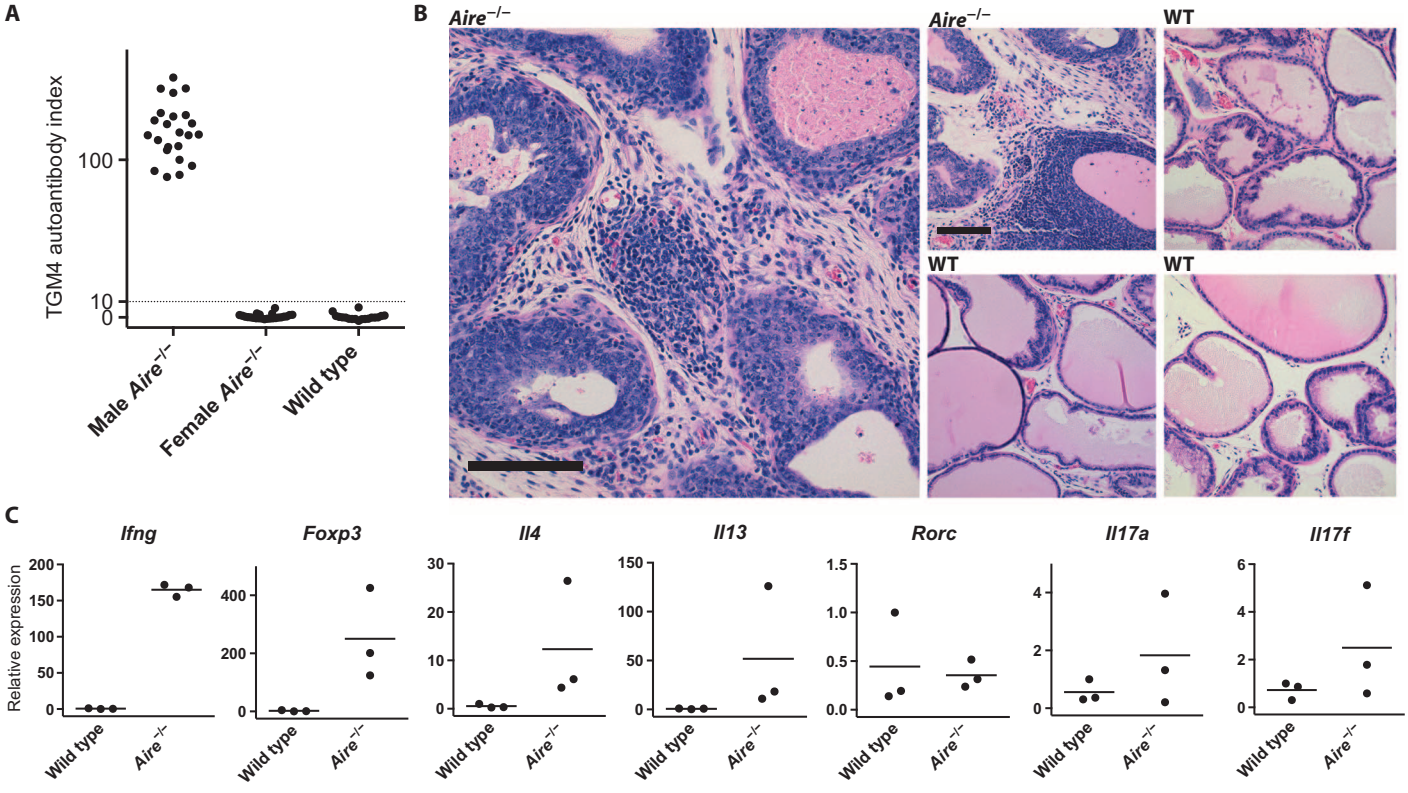

Fig. 6. (**A**) ***Aire*-deficient mice develop TGM4 autoantibodies and prostatitis.** Autoantibodies against murine TGM4 were measured in 66 mice of the C57Bl/6 strain, including 23 male *Aire*$^{-/-}$, 24 female *Aire*$^{-/-}$, 11 males of wild type, and 8 females of wild type (WT), and were detected in all male *Aire*$^{-/-}$ mice but were absent in all female and all wild-type mice. (**B**) The prostate histology was investigated in two *Aire*$^{-/-}$ mice with TGM4 autoantibodies and in three wild-type mice (all of NOD background), revealing marked signs of an active prostatitis in the *Aire*$^{-/-}$ and normal histology in wild-type mice. Scale bars, 500 μm. (**C**) To characterize the prostate-infiltrating T cells, markers for T_H1, T_H2, T_H17, or T_{reg} subsets were assessed in prostate tissue from three *Aire*$^{-/-}$ and three wild-type NOD mice. *Aire*$^{-/-}$ mice showed high expression of the T_H1-associated cytokine IFNγ and the T_{reg}-specific transcription factor Foxp3 compared to wild-type mice, whereas T_H2-associated cytokines (*Il4* and *Il13*) and T_H17-associated molecules (*Rorc*, *Il17a*, and *Il17f*) were not significantly increased in the *Aire*$^{-/-}$ mice, indicating a predominance of T_H1 and Treg cell subsets in prostatitis. qPCR results are standardized to β-actin and normalized to wild type. Bars indicate means.

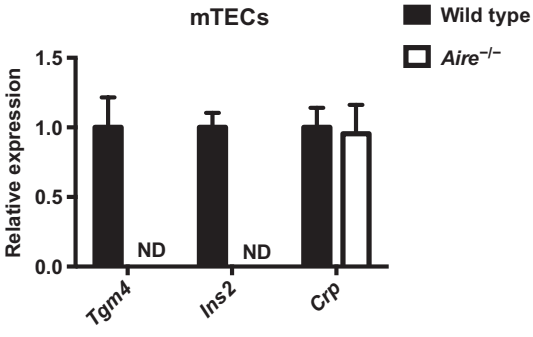

Fig. 7. ***Aire*-dependent expression of TGM4 in mTECs.** *Tgm4* mRNA levels were assessed by qPCR in mTECs from *Aire*$^{-/-}$ mice and wild-type mice of the C57Bl/6 strain and were compared with that of *Ins2*, an established *Aire*-dependent gene, and *Crp*, a gene known to be expressed in mTECs independently of *Aire*. TGM4 mRNA was detected in mTECs from wild-type mice but was undetectable in mTECs from *Aire*$^{-/-}$ mice. qPCR results are standardized to β_2-microglobulin (β_2M) and normalized to wild-type mTECs, with bars depicting means ± SD. ND, not detected.

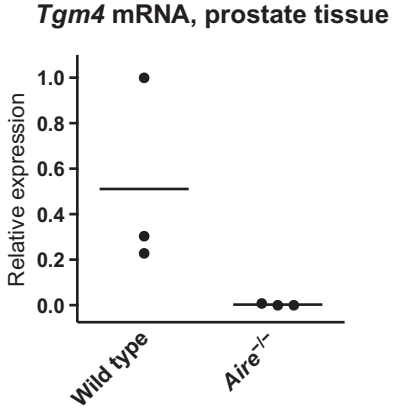

Fig. 8. ***Aire*-deficient mouse prostates lack TGM4.** *Tgm4* mRNA levels were assessed in prostate tissue from three *Aire*-deficient NOD mice with TGM4 autoantibodies and from three wild-type NOD mice. qPCR results are standardized to β-actin and normalized to wild type. Bars indicate means.

The restricted tissue distribution of TGM4, limited to the prostate gland of postpubertal males, allows for an exclusive observation to be made. As exemplified twice, first by comparing males and females and second by comparing males before and after pubertal onset, it was shown that autoantibodies only developed in the presence of the autoantigen despite a shared defect of central tolerance. These observations reveal an essential role of peripheral antigen presentation in driving the autoimmune response. Manipulation of peripheral antigen expression, through RNA interference or by other means, may be investigated as a therapeutic strategy in tissue-specific autoimmune disease.

MATERIALS AND METHODS

Study design

The aim of this study was to investigate disease mechanisms underlying subfertility in male APS1 patients by assessing the autoimmune response against male reproductive organs. The explorative and validation phases of the study were conducted using serum samples from APS1 patients and various control cohorts, and follow-up mechanistic studies were performed in the mouse model of APS1. A total of 93 APS1 patients were included in the study, which were divided into a discovery cohort of 27 males and 24 females and a replication cohort of 19 males and 23 females. The APS1 discovery cohort and a sex-matched healthy control group were subjected to a proteome-wide screen for autoantibodies. Autoantigens in male reproductive organs were identified by selecting for gender-imbalanced autoantibody signals. A novel candidate autoantigen, TGM4, was validated using an independent method (RLBA) and by reinvestigating the APS1 discovery cohort, assessing the APS1 replication cohort, and screening healthy and various disease control cohorts. None of the investigated sera were excluded after the analyses because of outlying results or of any other reason. The tissue distribution of TGM4 was validated on mRNA and protein levels in multiple human tissues by digital droplet PCR, immunohistochemistry, and immuno-rolling circle amplification. To assess the involvement of TGM4 immunity in the mouse model of APS1, previously collected serum samples from C57BI/6 and NOD mice were screened for autoantibodies against murine TGM4 using an RLBA. Prostate tissue histology was investigated in *Aire*-deficient and wild-type mice, and prostate-infiltrating immune cells were characterized by qPCR. To investigate the occurrence of *Aire*-dependent thymic presentation of TGM4, mRNA levels were assessed by qPCR in pooled mTECs from multiple *Aire*-deficient mice and pooled mTECs from multiple wild-type mice. Prostate levels of TGM4 were assayed in *Aire*-deficient and wild-type mice by qPCR.

Clinical subjects

Autoimmune polyendocrine syndrome type 1. Serum samples were obtained from Finnish, Norwegian, and Swedish patients with APS1. All individuals met the clinical diagnostic criteria for APS1, requiring two of the hallmark components: chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency, or at least one of the hallmark components in siblings or children of APS1 patients. Most of the patients had also been genotyped and demonstrated typical mutations in the *AIRE* gene (table S1). Chronic mucocutaneous candidiasis was defined as *Candida* infection of the oral mucosa, skin, or nails for a period of more than 3 months. Hypoparathyroidism was defined as plasma calcium concentration below 2.15 mM and elevated plasma phosphate concentration in combination with normal or low parathormone (PTH) concentration and normal renal function. Adrenal insufficiency was defined as subnormal serum cortisol in combination with elevated plasma adrenocorticotropic hormone (ACTH) concentration or deficient response to synthetic ACTH stimulation test (failure to reach 550 nM in 30 or 60 min). The patients had also been diagnosed with additional disease components, including alopecia, hypogonadism, vitiligo, type 1 diabetes mellitus, malabsorption, and pernicious anemia.

Control subjects. Serum samples were obtained from patients with chronic pelvic pain syndrome [cohorts described previously (*46*, *47*)], patients with biopsy confirmed inflammatory prostatitis and prostate cancer, and males with idiopathic infertility [previously described (*48*)]. Serum samples were also collected from patients with Addison's disease, primary Sjögren's syndrome, type 1 diabetes mellitus, autoimmune thyroiditis, and celiac disease. Blood donors were included as healthy controls. All healthy and disease control subjects were treated anonymously.

The project was approved by local ethical boards and was performed in accordance with the declarations of Helsinki. All patients and healthy subjects had given their informed consent for participation.

Mice

Aire-deficient mice were generated as previously described (*10*). All mice were housed in a pathogen-free barrier facility at the University of California San Francisco (UCSF), and all procedures were approved by the UCSF Institutional Animal Care Committee and Veterinary Services and adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Organs from mice were harvested, embedded in paraffin, and sectioned for hematoxylin and eosin staining. Sera were collected from mice that ranged in age between 40 days and 1 year.

Protein array screening

Protein arrays were probed and scanned according to Invitrogen's protocol for immune response biomarker profiling, and is briefly explained. Arrays, affinity reagents, and blocking buffer were purchased from Life Technologies [ProtoArray Human Protein Microarray v5.0 (PAH0525020, Life Technologies), Alexa Fluor 647 goat anti-human IgG (A21445, Life Technologies), and ProtoArray Blocking Buffer Kit (PA055, Life Technologies)]. Serum samples from APS1 patients (n = 51) and healthy blood donors (n = 21) were diluted 1:2000 in washing buffer. Incubations and washing steps were performed in a four-chamber tray on 50 rpm rotation at 4°C. The arrays were first incubated in blocking buffer for 1 hour, thereafter incubated for 90 min in 5 ml of diluted serum, and finally incubated for 90 min with Alexa Fluor 647 goat anti-human IgG antibody at a concentration of 1 μg/ml. The arrays were scanned using a GenePix 4000B microarray scanner. The GenePix Pro microarray (v6.1) software was used for alignment and data acquisition.

Statistical analyses

All statistical analyses of protein array data were performed on log-transformed intensities. T test was used to identify protein array targets that differed between patients and healthy controls, and Fisher's exact test was used to compare the frequency of autoantibody-positive male and female patients. Both tests were two-sided, and for the Fisher's exact test, the significance level $\alpha = 0.05$ was adopted after

the Bonferroni correction was used to account for multiple testing. The *t* test was used for ranking the targets, not for significance testing.

TGM4 radioligand binding assay

TGM4 autoantibodies were measured in serum by immunoprecipitation with radiolabeled TGM4 protein. Human TGM4 complimentary DNA (cDNA) (SC303287, OriGene) was cloned into pTNT vector (L5610, Promega) and was used for in vitro transcription and translation in the presence of [35S]methionine (Promega TNT Systems). Immunoprecipitation was conducted in 96-well filtration plates (Millipore). A positive standard, represented by an APS1 patient serum with TGM4-specific autoantibodies, and a negative standard, 4% bovine serum albumin (BSA), were included on each plate. All serum samples were analyzed in duplicate. Radiolabeled TGM4 protein (40,000 cpm) and 2.5 μl of serum sample were added to each well, and samples were incubated overnight. Serum antibodies were then immobilized to protein A Sepharose (nProtein A Sepharose 4 Fast Flow, GE Healthcare) during 45 min of incubation. The plates were washed multiple times and then dried. Scintillation solution was added and radioactivity was measured in a microbeta counter (1450 MicroBeta TriLux, Wallac). Index values were calculated according to the following equation: [(sample value/negative standard)/(positive standard value/negative standard value)] × 100. Autoantibodies against tissue transglutaminase, 21-hydroxylase, GAD65, 17-hydroxylase, side-chain cleavage enzyme, tyrosine hydroxylase, tryptophan hydroxylase, aromatic L amino acid decarboxylase, CYP1A2, and murine TGM4 (MC220389, OriGene) were analyzed in the same manner.

Protein sequence comparison between TGM4 and TGM2

Protein sequences of human TGM4 [National Center for Biotechnology Information (NCBI) Reference Sequence: NP_003232.2] and TGM2 (NCBI Reference Sequence: NP_004604.2) were compared using the Basic Local Alignment Search Tool http://blast.ncbi.nlm.nih.gov/Blast.cgi

Phylogenetic tree of the transglutaminase family

Protein sequences of members of the human transglutaminase family were aligned using T-Coffee with default settings. The UPGMA (unweighted pair group method with arithmetic mean) algorithm in Clustal X was used for hierarchic clustering.

Digital droplet PCR expression assay

Total RNA was purchased from OriGene and was converted to cDNA using Life Technologies' Double-Stranded cDNA Synthesis Kit and protocol with random hexamers (N8080127) for the first-strand synthesis. Bio-Rad's ddPCR Supermix for Probes (186-3040) was used, and droplet generation was performed according to Bio-Rad's standard protocol with TaqMan probes to TGM4 and β_2M as controls (4331182 and 4448484, Life Technologies). The samples were run on a QuantaLife ddPCR machine, and QuantaSoft software was used to collect and analyze event data and generate figures.

TGM4 immunohistochemistry on human tissue microarrays

Tissue microarrays (TMA) were constructed from human formalin-fixed paraffin-embedded tissues according to earlier description (*49*). Immunohistochemistry was carried out as detailed previously and is described in brief. A TMA was stained with goat TGM4 antibody (sc-55791, LOTA1312, Santa Cruz Biotechnology), using the Autostainer 480 (Thermo Fisher Scientific). Incubations were done at room temperature, and all reagents were applied at a volume of 300 μl per slide. The slide was first incubated with Ultra V Block (TA-125-UB, Thermo Fisher Scientific) for 5 min, and thereafter incubated with anti-TGM4 at a dilution of 1:50 for 30 min. Next, the slide was incubated with labeled horseradish peroxidase polymer for 30 min, followed by 3,3′-diaminobenzidine solution for 2 × 5 min. The slide was counterstained in Mayer's hematoxylin (01820, Histolab) for 5 min using the Autostainer XL (Leica), and then rinsed in lithium carbonate water (diluted 1:5 from saturated solution) for 1 min. The slide was dehydrated in graded ethanol and then coverslipped (PERTEX, Histolab) using an automated glass coverslipper (CV5030, Leica). The TMA was scanned using the automated scanning system Aperio XT (Aperio Technologies).

TGM4 protein detection by immuno-rolling circle amplification in human TMAs

Slide preparation. TMA sections were deparaffinized at 45°C overnight (about 20 hours), followed by immersions in xylene for 15 plus 5 min, in 99.9% ethanol for 2 × 2 min, in 95% ethanol for 2 × 2min, and finally in 70% ethanol for 2 min and double-distilled water (ddH_2O) for 2 min (all immersions at room temperature). Slides were then placed in 250 ml of 1 × Target Retrieval Solution Citrate (pH 6; S2369, Dako) in a staining jar placed in a pressure boiler containing 600 ml of Milli-Q–H_2O (MQ-H_2O) with the following program: 5 min at 125°C, 20 min at 80°C, followed by a cooldown to 40°C. Slides were then rinsed three times in ddH_2O and washed twice in 1 × tris-buffered saline (TBS) for 2 min. Areas around tissues were carefully dried, a hydrophobic border was traced around the tissues with an ImmEdge pen (H-4000, Vector Laboratories), and the tissues were immediately covered with 1 × TBS to avoid drying.

Immuno-rolling circle amplification. Blocking solution, antibody diluent, and secondary antibody were provided in Duolink In Situ PLA Probe Anti-Goat PLUS (DUO92003, Sigma-Aldrich). All incubations were carried out at 37°C in a humidity chamber, and all reagents were applied at a volume of 100 μl per slide, unless stated otherwise.

All washes were performed in 70 ml of 1 × TBS containing 0.05% Tween 20 for 2 × 5 min. Five drops of blocking solution were added to each section, and slides were incubated for 1 hour. Goat anti-TGM4 antibody (sc-55791, LOTA1312, Santa Cruz Biotechnology) was diluted 1:200 in antibody diluent, blocking solution was tapped off from the slides, and 100 μl of antibody mix was added to each sample. The slides were incubated at 4°C overnight. The slides were then washed once, Duolink anti-goat plus probe (diluted 1:5 in antibody diluent) was added, and the slides were incubated for 1 hour followed by washing. Padlock probe targeting the probe's single-stranded DNA arm (5′-P-GTTCTGTCATACAGTGAATGCGAGTC CGTCTAAGAGAGTAG-TACAGCAGCCGTCAAGAGTGTCTA-3′) was added to the slides in a mix containing BSA (0.25 mg/ml), 10 mM tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 25 mM NaCl, 0.1% Tween 20, and 0.125 μM padlock probe. The slides were incubated for 30 min and then washed. The padlock probe was ligated using T4 DNA ligase (EL0011, Thermo Scientific) at a concentration of 0.05 U/μl in 1 × T4 DNA ligation buffer and incubated for 30 min. Rolling circle amplification and detection were performed by adding 100 μl of amplification mix [BSA (0.25 mg/ml), polyadenylic acid (7.5 ng/ml), 1 × ϕ29 reaction buffer, 0.25 mM deoxynucleotide triphosphate, 0.025 μM detection oligo (5′-Cy3-CAGTGAATGCGAGTCCGTCTmUmUmUmU-3′, where mU is 2′-*O*-methyl RNA that is used to block the polymerase's exonuclease activity), Hoechst 33342 (0.1 mg/ml; H1399, Life Technologies), and ϕ29

DNA polymerase (0.25 U/μl; EP0092, Thermo Scientific)] to the slides followed by incubation for 100 min. The slides were then washed 2 × 10 min in 1 × TBS, followed by 30-min rinsing in MQ-H_2O, and were air-dried. Slowfade Gold Antifade Reagent (40 μl; S36940, Life Technologies) was added to the slides, which were then covered with coverslips. Resulting signals were visualized using an Axioplan 2 epifluorescence microscope (Zeiss); pictures were acquired using AxioVision 4.8 (Zeiss).

Expression analyses in mTECs

Thymic epithelial cells were isolated as previously described (*50*). Briefly, thymi from six to seven mice were minced and digested with deoxyribonuclease I and Liberase TM (Roche) before gradient centrifugation with Percoll PLUS (GE Healthcare). Enriched stromal cells were stained with CD11c, CD45, EpCAM, I-A^b, and Ly51 (BioLegend). Cell sorting was performed using a BD FACSAria III cell sorter. Sorted mTECs, defined as DAPI$^-$, CD11c$^-$, CD45$^-$, EpCAM$^+$, Ly51$^-$, MHCII^{hi}, were collected in a 1:1 mixture of Dulbecco's minimum essential medium and fetal bovine serum, and RNA was extracted using an RNeasy Micro Plus Kit (Qiagen). cDNA was synthesized using a SuperScript III kit (Invitrogen). Applied Biosystems TaqMan gene expression assays were used for all targets. All targets were standardized to β_2M signals.

Expression analyses in mouse prostates

Prostates from *Aire*-deficient and wild-type mice of the NOD strain and ages 42 to 46 days were harvested, and RNA was isolated using the RNeasy Mini Kit (Qiagen). Two micrograms of total RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). qPCR was conducted using mouse primers for *Tgm4, Ifng, Il4, Il13, Rorc, Il17a, Il17f*, and *Foxp3* (Applied Biosystems), and expression levels were standardized to β-actin (assay ID 4352933E) and were normalized to wild type.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/7/292/292ra101/DC1
Fig. S1. Confirmation of TGM4 autoantibodies using an RLBA.
Fig. S2. TGM4 mRNA expression in multiple human tissues, detected by digital droplet PCR.
Fig. S3. TGM4 protein expression in multiple human tissues, detected by immunohistochemistry.
Fig. S4. TGM4 protein expression in multiple human tissues, detected by immuno-rolling circle amplification.
Fig. S5. Male *Aire*-deficient NOD mice display TGM4 autoantibodies.
Fig. S6. Male wild-type NOD mice display TGM4 autoantibodies.
Table S1. Characterization of APS1 patients.
Table S2. Autoantibodies in young male APS1 patients.
Table S3. TGM4 autoantibodies first appear during pubertal age.
Table S4. mRNA expression in mouse prostates.
Table S5. TGM4 mRNA expression in mTECs.

REFERENCES AND NOTES

1. C. Krausz, Male infertility: Pathogenesis and clinical diagnosis. *Best Pract. Res. Clin. Endocrinol. Metab.* **25**, 271–285 (2011).
2. B. D. Anawalt, Approach to male infertility and induction of spermatogenesis. *J. Clin. Endocrinol. Metab.* **98**, 3532–3542 (2013).
3. P. J. Stahl, D. S. Stember, M. Goldstein, Contemporary management of male infertility. *Annu. Rev. Med.* **63**, 525–540 (2012).
4. S. Alshahrani, J. McGill, A. Agarwal, Prostatitis and male infertility. *J. Reprod. Immunol.* **100**, 30–36 (2013).
5. M. Fraczek, M. Kurpisz, Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. *J. Androl.* **28**, 325–333 (2007).
6. M. Marconi, A. Pilatz, F. Wagenlehner, T. Diemer, W. Weidner, Impact of infection on the secretory capacity of the male accessory glands. *Int. Braz. J. Urol.* **35**, 299–309 (2009).
7. R. D. Motrich, J. P. Mackern-Oberti, M. Maccioni, V. E. Rivero, Effects of autoimmunity to the prostate on the fertility of the male rat. *Fertil. Steril.* **91**, 2273–2280 (2009).
8. T. Kullisaar, S. Türk, M. Punab, R. Mändar, Oxidative stress—Cause or consequence of male genital tract disorders? *Prostate* **72**, 977–983 (2012).
9. P. Ahonen, S. Myllärniemi, I. Sipilä, J. Perheentupa, Clinical variation of autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) in a series of 68 patients. *N. Engl. J. Med.* **322**, 1829–1836 (1990).
10. M. S. Anderson, E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, D. Mathis, Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395–1401 (2002).
11. M. H. Cheng, M. S. Anderson, Monogenic autoimmunity. *Annu. Rev. Immunol.* **30**, 393–427 (2012).
12. M. Alimohammadi, N. Dubois, F. Sköldberg, Å. Hallgren, I. Tardivel, H. Hedstrand, J. Haavik, E. S. Husebye, J. Gustafsson, F. Rorsman, A. Meloni, C. Janson, B. Vialettes, M. Kajosaari, W. Egner, R. Sargur, F. Pontén, Z. Amoura, A. Grimfeld, F. De Luca, C. Betterle, J. Perheentupa, O. Kämpe, J.-C. Carel, Pulmonary autoimmunity as a feature of autoimmune polyendocrine syndrome type 1 and identification of KCNRG as a bronchial autoantigen. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4396–4401 (2009).
13. J. Perheentupa, APS-I/APECED: The clinical disease and therapy. *Endocrinol. Metab. Clin. North Am.* **31**, 295–320, (2002).
14. C. Ramsey, O. Winqvist, L. Puhakka, M. Halonen, A. Moro, O. Kämpe, P. Eskelin, M. Pelto-Huikko, L. Peltonen, Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum. Mol. Genet.* **11**, 397–409 (2002).
15. F.-X. Hubert, S. A. Kinkel, P. E. Crewther, P. Z. Cannon, K. E. Webster, M. Link, R. Uibo, M. K. O'Bryan, A. Meager, S. P. Forehan, G. K. Smyth, L. Mittaz, S. E. Antonarakis, P. Peterson, W. R. Heath, H. S. Scott, Aire-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype. *J. Immunol.* **182**, 3902–3918 (2009).
16. E. Kekäläinen, N. Pöntynen, S. Meri, T. P. Arstila, H. Jarva, Autoimmunity, not a developmental defect, is the cause for subfertility of autoimmune regulator (Aire) deficient mice. *Scand. J. Immunol.* **81**, 298–304 (2015).
17. Y. Y. Setiady, K. Ohno, E. T. Samy, H. Bagavant, H. Qiao, C. Sharp, J. X. She, K. S. Tung, Physiologic self antigens rapidly capacitate autoimmune disease-specific polyclonal CD4$^+$ CD25$^+$ regulatory T cells. *Blood* **107**, 1056–1062 (2006).
18. Q.-G. Ruan, K. Tung, D. Eisenman, Y. Setiady, S. Eckenrode, B. Yi, S. Purohit, W.-P. Zheng, Y. Zhang, L. Peltonen, J.-X. She, The autoimmune regulator directly controls the expression of genes critical for thymic epithelial function. *J. Immunol.* **178**, 7173–7180 (2007).
19. Y. Hou, J. DeVoss, V. Dao, S. Kwek, J. P. Simko, D. G. McNeel, M. S. Anderson, L. Fong, An aberrant prostate antigen-specific immune response causes prostatitis in mice and is associated with chronic prostatitis in humans. *J. Clin. Invest.* **119**, 2031–2041 (2009).
20. H. J. Dubbink, L. de Waal, R. van Haperen, N. S. Verkaik, J. Trapman, J. C. Romijn, The human prostate-specific transglutaminase gene (*TGM4*): Genomic organization, tissue-specific expression, and promoter characterization. *Genomics* **51**, 434–444 (1998).
21. H. J. Dubbink, N. S. Verkaik, P. W. Faber, J. Trapman, F. H. Schröder, J. C. Romijn, Tissue-specific and androgen-regulated expression of human prostate-specific transglutaminase. *Biochem. J.* **315**, 901–908 (1996).
22. M. D. Dean, Genetic disruption of the copulatory plug in mice leads to severely reduced fertility. *PLOS Genet.* **9**, e1003185 (2013).
23. D. C. Mukherjee, A. K. Agrawal, R. Manjunath, A. B. Mukherjee, Suppression of epididymal sperm antigenicity in the rabbit by uteroglobin and transglutaminase in vitro. *Science* **219**, 989–991 (1983).
24. G. Paonessa, S. Metafora, G. Tajana, P. Abrescia, A. De Santis, V. Gentile, R. Porta, Transglutaminase-mediated modifications of the rat sperm surface in vitro. *Science* **226**, 852–855 (1984).
25. R. Porta, C. Esposito, A. De Santis, A. Fusco, M. Iannone, S. Metafora, Sperm maturation in human semen: Role of transglutaminase-mediated reactions. *Biol. Reprod.* **35**, 965–970 (1986).
26. G. Peluso, R. Porta, C. Esposito, M. A. Tufano, R. Toraldo, M. L. Vuotto, G. Ravagnan, S. Metafora, Suppression of rat epididymal sperm immunogenicity by a seminal vesicle secretory protein and transglutaminase both in vivo and in vitro. *Biol. Reprod.* **50**, 593–602 (1994).
27. W. Dieterich, T. Ehnis, M. Bauer, P. Donner, U. Volta, E. O. Riecken, D. Schuppan, Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat. Med.* **3**, 797–801 (1997).
28. M. Sárdy, S. Kárpáti, B. Merkl, M. Paulsson, N. Smyth, Epidermal transglutaminase (TGase 3) is the autoantigen of dermatitis herpetiformis. *J. Exp. Med.* **195**, 747–757 (2002).
29. M. Hadjivassiliou, P. Aeschlimann, A. Strigun, D. S. Sanders, N. Woodroofe, D. Aeschlimann, Autoantibodies in gluten ataxia recognize a novel neuronal transglutaminase. *Ann. Neurol.* **64**, 332–343 (2008).
30. M. Franchini, F. Frattini, S. Crestani, C. Bonfanti, Acquired FXIII inhibitors: A systematic review. *J. Thromb. Thrombolysis* **36**, 109–114 (2013).
31. J. L. Thielen, K. G. Volzing, L. S. Collier, L. E. Green, D. A. Largaespada, P. C. Marker, Markers of prostate region-specific epithelial identity define anatomical locations in the mouse

prostate that are molecularly similar to human prostate cancers. *Differentiation* **75**, 49–61 (2007).
32. K.-C. Ho, V. E. Quarmby, F. S. French, E. M. Wilson, Molecular cloning of rat prostate transglutaminase complementary DNA. The major androgen-regulated protein DP1 of rat dorsal prostate and coagulating gland. *J. Biol. Chem.* **267**, 12660–12667 (1992).
33. D. A. Goldfarb, B. S. Stein, M. Shamszadeh, R. O. Petersen, Age-related changes in tissue levels of prostatic acid phosphatase and prostate specific antigen. *J. Urol.* **136**, 1266–1269 (1986).
34. J. G. Vieira, S. K. Nishida, A. B. Pereira, R. F. Arraes, I. T. Verreschi, Serum levels of prostate-specific antigen in normal boys throughout puberty. *J. Clin. Endocrinol. Metab.* **78**, 1185–1187 (1994).
35. C. Pritchard, B. Mecham, R. Dumpit, I. Coleman, M. Bhattacharjee, Q. Chen, R. A. Sikes, P. S. Nelson, Conserved gene expression programs integrate mammalian prostate development and tumorigenesis. *Cancer Res.* **69**, 1739–1747 (2009).
36. G. Penna, S. Amuchastegui, C. Cossetti, F. Aquilano, R. Mariani, N. Giarratana, E. De Carli, B. Fibbi, L. Adorini, Spontaneous and prostatic steroid binding protein peptide-induced autoimmune prostatitis in the nonobese diabetic mouse. *J. Immunol.* **179**, 1559–1567 (2007).
37. J. J. Devoss, A. K. Shum, K. P. Johannes, W. Lu, A. K. Krawisz, P. Wang, T. Yang, N. P. Leclair, C. Austin, E. C. Strauss, M. S. Anderson, Effector mechanisms of the autoimmune syndrome in the murine model of autoimmune polyglandular syndrome type 1. *J. Immunol.* **181**, 4072–4079 (2008).
38. O. Winqvist, J. Gustafsson, F. Rorsman, F. A. Karlsson, O. Kämpe, Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addison's disease. *J. Clin. Invest.* **92**, 2377–2385 (1993).
39. A. Tsatsoulis, S. M. Shalet, Antisperm antibodies in the polyglandular autoimmune (PGA) syndrome type I: Response to cyclical steroid therapy. *Clin. Endocrinol.* **35**, 299–303 (1991).
40. A. Peter, H. Lilja, Å Lundwall, J. Malm, Semenogelin I and semenogelin II, the major gel-forming proteins in human semen, are substrates for transglutaminase. *Eur. J. Biochem.* **252**, 216–221 (1998).
41. S. E. Iismaa, B. M. Mearns, L. Lorand, R. M. Graham, Transglutaminases and disease: Lessons from genetically engineered mouse models and inherited disorders. *Physiol. Rev.* **89**, 991–1023 (2009).
42. L. Lorand, R. M. Graham, Transglutaminases: Crosslinking enzymes with pleiotropic functions. *Nat. Rev. Mol. Cell Biol.* **4**, 140–156 (2003).
43. R. Nissinen, L. Paimela, H. Julkunen, P. J. Tienari, M. Leirisalo-Repo, T. Palosuo, O. Vaarala, Peptidylarginine deiminase, the arginine to citrulline converting enzyme, is frequently recognized by sera of patients with rheumatoid arthritis, systemic lupus erythematosus and primary Sjögren syndrome. *Scand. J. Rheumatol.* **32**, 337–342 (2003).
44. F. C. Santos, R. P. Leite, A. M. Custódio, K. P. Carvalho, L. H. Monteiro-Leal, A. B. Santos, R. M. Góes, H. F. Carvalho, S. R. Taboga, Testosterone stimulates growth and secretory activity of the female prostate in the adult gerbil (*Meriones unguiculatus*). *Biol. Reprod.* **75**, 370–379 (2006).
45. C. V. Obiezu, E. J. Giltay, A. Magklara, A. Scorilas, L. J. Gooren, H. Yu, D. J. Howarth, E. P. Diamandis, Serum and urinary prostate-specific antigen and urinary human glandular kallikrein concentrations are significantly increased after testosterone administration in female-to-male transsexuals. *Clin. Chem.* **46**, 859–862 (2000).
46. R. D. Motrich, M. Maccioni, R. Molina, A. Tissera, J. Olmedo, C. M. Riera, V. E. Rivero, Presence of INFγ-secreting lymphocytes specific to prostate antigens in a group of chronic prostatitis patients. *Clin. Immunol.* **116**, 149–157 (2005).
47. D. Lundh, H. Hedelin, K. Jonsson, M. Gifford, D. Larsson, Assessing chronic pelvic pain syndrome patients: Blood plasma factors and cortisol saliva. *Scand. J. Urol.* **47**, 521–528 (2013).
48. L. E. Murphy, J. L. Mills, A. M. Molloy, C. Qian, T. C. Carter, H. Strevens, D. Wide-Swensson, A. Giwercman, R. J. Levine, Folate and vitamin B_{12} in idiopathic male infertility. *Asian J. Androl.* **13**, 856–861 (2011).
49. C. Kampf, I. Olsson, U. Ryberg, E. Sjöstedt, F. Pontén, Production of tissue microarrays, immunohistochemistry staining and digitalization within the human protein atlas. *J. Vis. Exp.* **63**, e3620 (2012).
50. T. C. Metzger, I. S. Khan, J. M. Gardner, M. L. Mouchess, K. P. Johannes, A. K. Krawisz, K. M. Skrzypczynska, M. S. Anderson, Lineage tracing and cell ablation identify a post-Aire-expressing thymic epithelial cell population. *Cell Rep.* **5**, 166–179 (2013).

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