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(54) **Titre : COMPOSITIONS ET METHODES POUR LA PROPHYLAXIE ET/OU LA THERAPIE DE TROUBLES QUI SONT EN CORRELATION AVEC LA VARIANTE 2 DE DENND1A**

(54) **Title: COMPOSITIONS AND METHODS FOR PROPHYLAXIS AND/OR THERAPY OF DISORDERS THAT CORRELATE WITH DENND1A VARIANT 2**

(57) **Abrégé/Abstract:**

Phannaceutical compositions and methods for the treatment of DENNDI A. V2 related disorders, such as PCOS, are provided. In particular, humanized and mouse monoclonal antibodies specific for DENNDI A. V2 and methods for using the same are provided.

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COMPOSITIONS AND METHODS FOR PROPHYLAXIS AND/OR THERAPY OF DISORDERS THAT CORRELATE WITH DENND1A VARIANT 2

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is related to U.S. Provisional application No. 61/906,078, filed on November 19, 2013 and US Provisional Application no. 62/042,852, filed on August 28, 2014, the disclosures of each of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to disorders that correlate with expression of DENND1A.V2 (DENN/MADD domain containing 1A variant 2) mRNA and/or protein, including without limitation polycystic ovary syndrome (PCOS), and more specifically to compositions and methods for prophylaxis and/or therapy of these disorders.

BACKGROUND OF THE INVENTION

[0003] Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies that affects 5-7% of reproductive age women world-wide [1]. It is associated with hyperandrogenemia/hyperandrogenism, anovulation, infertility, and a characteristic ovarian morphology consisting of multiple small subcortical follicular "cysts" embedded in bilaterally enlarged ovaries [2-5]. The presence of an elevated level of circulating testosterone results primarily from increased production of androgens by the ovaries, and is a classical endocrine phenotype of women with PCOS. Although there has been debate about the diagnostic criteria for PCOS, hyperandrogenemia/hyperandrogenism and anovulation, not explained by other causes, is a hallmark of the disorder, and is included as a key element in all "consensus" diagnosis schemes [6-10]. There is consensus that the ovarian theca cells are the primary source of excess androgen biosynthesis in women with PCOS [11-13]. Studies on freshly isolated theca tissue, or cultures of theca cells derived from normal and PCOS women have demonstrated that PCOS theca secretes greater amounts of androgen than theca tissue or cells from regularly ovulating women [12, 14-19]. Increased thecal androgen biosynthesis in PCOS theca cells results from increased expression of the key enzymes involved in androgen biosynthesis, cytochrome P450 17 alpha hydroxylase (encoded by the *CYP17A1* gene) and cytochrome P450 cholesterol side chain cleavage (encoded by the *CYP11A1* gene) [15-17, 20].

SUMMARY OF THE INVENTION

[0004] The development of conditions to propagate theca cells isolated from individual, size-matched follicles from ovaries of normal cycling and PCOS women, provided the first evidence that successively passaged PCOS theca cells retain the ability to produce augmented levels of androgens and progesterone compared normal theca cells [15, 16, 21]. This increase in androgen and progesterone biosynthesis in PCOS theca cells has been attributed, in part, to increased *CYP17A1* and *CYP11A1* gene transcription and RNA stability [17, 20, 22]. Molecular characterization of normal and PCOS theca cells from multiple individuals by microarray analysis and quantitative PCR also established that normal and PCOS cells have distinctive molecular signatures [16, 23, 24]. These findings are consistent with the notion of an intrinsic abnormality in PCOS theca that promotes hypersecretion of androgens in response to tropic stimulation. The theca cell culture system provides a unique platform for identifying the biochemical and molecular mechanisms underlying genetic abnormalities in PCOS women.

[0005] In the present disclosure, data is presented which demonstrates that an alternatively spliced, truncated form of DENND1A, DENND1A.V2 [25, 26], is differentially expressed in normal and PCOS theca cells. The data demonstrates that DENND1A.V2 contributes to the pathophysiological state of increased *CYP17A1* and *CYP11A1* gene expression and augmented androgen and progestin biosynthesis by PCOS theca cells [17, 20, 22]. Moreover, data presented herein demonstrates that targeting DENND1A.V2 using either shRNA or antibody-based approaches can suppress *CYP17A1* and *CYP11A1* gene expression and the increased androgen production exhibited by PCOS theca cells. Thus, inhibiting the increased androgen expression that is a hallmark of PCOS will have a prophylactic and/or therapeutic effect in PCOS patients, as well as in individuals who have other disorders that are positively associated with DENND1A.V2 expression.

[0006] One aspect of the invention comprises administering to a subject in need thereof a composition comprising an effective amount of a DENND1A.V2 targeting agent such that a prophylactic and/or therapeutic benefit is attained. Exemplary targeting agents include, but are not limited to, antibodies or antigen binding fragments and RNAi, shRNA or other nucleotide containing compositions. In some embodiments, the subject is in need of treatment for any disorder that is positively correlated with DENND1A.V2 expression. In some embodiments the

disorder is correlated with DENND1A.V2 that is increased relative to a reference. In additional embodiments, the individual is at risk for, suspected of having, or has been diagnosed with PCOS.

[0007] Other aspects of the invention relate to pharmaceutical compositions comprising an antibody or an antigen binding fragment thereof that specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 (DENN/MADD domain containing 1A variant 1) protein. Examples of antigen binding fragments include, but are not limited to, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fd fragments, Fv fragments, scFv fragments, and combinations thereof. In preferred embodiments, the antibody or antigen binding fragment specifically recognizes at least one epitope present in the distinct DENND1A.V2 C-terminal amino acid sequence: NTIATPATLHILQKSITHFAAKFPTRGWTSSSH (SEQ ID NO:5).

[0008] Other features and advantages of the present invention will be set forth in the description of invention that follows, and in part will be apparent from the description or may be learned by practice of the invention. The invention will be realized and attained by the compositions and methods particularly pointed out in the written description and claims hereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Figure 1A-F. DENND1A.V2 protein is increased in PCOS theca cells. Representative Western analysis of ~62 kD DENND1A.V2 and ~112kD DENND1A.V1 in whole cell extracts from normal and PCOS theca cells treated in the absence (-) and presence (+) of 20 μM forskolin. Total mTOR was used for protein normalization (Fig 1A). Quantitative data from Western analyses from theca cells isolated from 5 normal cycling and 5 PCOS subjects, presented as the mean ± SEM, demonstrated that DENND1A.V2 protein was increased in both basal and forskolin-stimulated (*, $P< 0.01$) PCOS theca cells as compared to normal theca cells (Fig 1B). As shown in Fig 1C, DENND1A.V1 was increased by forskolin treatment in normal theca cells (*, $P< 0.01$). Forskolin-stimulated DENND1A.V1 was decreased in PCOS theca cells as compared normal cells (**, $P< 0.01$) (Fig 1C). The ratio of DENND1A.V2/V1 was increased (*, $P< 0.01$) in PCOS theca cells under control and forskolin-stimulated conditions (Fig 1D). In Fig 1E, Western analyses was performed to evaluate the efficacy of a rabbit polyclonal antibody, generated against the 21 amino acid peptide (QKSITHFAAKFPTRGWTSSSH)(SEQ NO:6) that is specific to DENND1A.V2, using whole

cell extracts from normal and PCOS theca cells treated as described above. As shown in Fig 1E, representative Western Blot analysis demonstrated an increase in 62 kD DENND1A.V2 in PCOS theca cells treated under both basal and forskolin-stimulated conditions. Total mTOR was used for protein normalization. As shown in Fig 1F, cumulative analysis of whole cell lysates harvested from theca cells isolated from 4 independent normal and 4 independent PCOS women, demonstrated that DENND1A.V2 protein is significantly increased in PCOS theca cells as compared to normal theca cells under both control and forskolin-stimulated conditions (*$P < 0.01$). Forskolin treatment did not appear to affect DENND1A.V2 protein accumulation in normal or PCOS theca cells.

[00010] **Figure 2A-B. Immunohistochemical localization of DENND1A.V2 in normal cycling and PCOS ovary.** In Fig 2A, DENND1A.V2 protein was localized in the theca interna of the ovarian follicles, and was increased in PCOS theca (bottom) as compared to normal theca (top). In Fig 2B, DENND1A.V2 staining in PCOS theca and granulosa cells is presented in the left panel (40X). Staining, primarily in the PCOS theca cell nuclei, cytoplasm, and cell membrane, is shown in the right panel (100X under oil).

[00011] **Figure 3A-B. DENND1A.V2 mRNA abundance is increased in PCOS theca cells, and is correlated with increased androgen production.** DENND1A.V2 (Fig 3A) mRNA abundance was compared in theca cells propagated from 6 individual normal and 6 individual PCOS women that were treated in the absence (C) and presence (F) of 20 μM forskolin for 16 h, using qRT-PCR. DENND1A.V2 mRNA was significantly increased under basal (*, $P<0.05$) and forskolin (**, $P<0.01$) stimulated conditions in PCOS theca cells, as compared to normal cells (Fig 3B). Both control (C) and forskolin (F) -stimulated DHEA accumulation from the 6 normal and 6 PCOS women's theca cell preparations were compared with DENND1A.V2 mRNA under the same conditions (Fig 3B).

[00012] **Figure 4. DENND1A Variant 2 mRNA is increased in urine exosomes isolated from PCOS women as compared to normal women.** Comparison of DENND1A.V2 RNA accumulation in exosomal mRNA purified and isolated from mid-day urine obtained from isolated from 5 normal cycling and 6 PCOS women (*, $P < 0.001$) using real-time qPCR analysis.

[00013] **Figure 5A-E. Forced expression of DENND1A.V2 in normal theca cells results in augmented androgen and progestin production.** To examine the effects of forced

expression DENND1A.V2 on androgen biosynthesis in normal theca cells, DHEA production by representative normal theca cells infected with 0.3, 1.0, 3.0, and 10 pfu/cell of either empty (Null) or DENND1A.V2 adenovirus, treated in the absence (C) or presence (F) of 20 μM forskolin for 72 h is presented in Fig 5A. As shown, DENND1A.V2 adenoviral infection and forced DENND1A.V2 expression, increases forskolin-stimulated DHEA production in normal theca cells, compared to control Null adenovirus. In subsequent studies normal theca cells were infected with either 3 pfu/cell of DENND1A.V2, or control, Null adenovirus and treated in the absence (C) or presence of (F) of 20 μM forskolin for 72h. DHEA (Fig 5B), 17OHP4 (Fig 5C), T (Fig 5D) and Progesterone (Fig 5E), were then measured and normalized by cell count. As shown, DENND1A.V2 adenovirus infection significantly increased basal 17OHP4 (Fig 5C; *, $P < 0.01$), T (Fig 5D; *, $P < 0.05$), and P4 (Fig 5E; *, $P < 0.05$) accumulation compared with control Null adenovirus. In addition, DENND1A.V2 adenovirus infection significantly increased forskolin-stimulated DHEA (Fig 5B; *, $P < 0.001$), 17OHP4 (Fig 5C; *, $P < 0.001$), and P4 (Fig 5E; *, $P < 0.001$) compared with control Null adenovirus. Thus forced expression of DENND1A.V2 in normal theca cells augments androgen and progesterone biosynthesis, and promotes a PCOS phenotype.

[00014] **Figure 6A-D. Forced expression of DENND1A.V2 in normal theca cells results in augmented CYP17 and CYP11A1 mRNA accumulation, as well as *CYP17A1* and *CYP11A1* promoter regulation.** To examine the effects of DENND1A.V2 on CYP17 and CYP11A1 mRNA accumulation, normal theca cells were infected either 3 pfu/cell of DENND1A.V2, or control, Null adenovirus and treated in the absence (C; control) or presence of (F; forskolin) of 20 μM forskolin for 16h. Following RNA isolation, CYP17 and CYP11A1 mRNA was quantitated by qRT-PCR and normalized by TBP mRNA. As shown, forced expression of DENND1A.V2 in normal theca cells, significantly increased forskolin-stimulated CYP17 mRNA (Fig 6A; *, $P< 0.01$) and CYP11A1 mRNA (Fig 6B; *, $P< 0.05$) accumulation. To examine the effects of DENND1A.V2 on *CYP17A1* transcription, normal theca cells were transfected with a *CYP17A1* promoter gene plasmid *(-770 CYP17A1*/LUC), and infected with DENND1A V2 or Null adenovirus (Fig 6C). As shown in Fig 6C, DENND1A.V2 adenovirus infection increases both basal (*, $P<0.05$) and forskolin-stimulated (**, $P<0.05$) *-770CYP17A1* promoter activity, as compared to Null control adenovirus. In Fig 6D, a *CYP11A1* promoter construct *(-160/-90 CYP11A1*/LUC) which confers increased *CYP11A1* expression in PCOS

theca cells to examine the effects of DENND1A.V2 on *CYP11A1* transcription in theca cells. Normal theca cells were transfected with the *-160/-90 CYP11A1*/LUC plasmid with an DENND1A.V2/pCMV-XL4 or control pCMV-XL4 plasmid. As shown in Fig 6D, DENND1A.V2 significantly increased forskolin-stimulated (*, P<0.001) *-160/-90 CYP11A1/LUC* promoter activity as compared to empty plasmid. These combined data demonstrate that DENND1A.V2 induces increased transcriptional activation of *CYP17A1* and *CYP11A1* gene expression.

[00015] **Figure 7A-F. Knock-down of DENND1A.V2 in PCOS theca cells results in a significant reduction in *CYP17A1* and *CYP11A1* expression, and decreased androgen and progestin biosynthesis.** Knockdown of endogenous DENND1A.V2 in PCOS theca cells, following transfection of PCOS theca cells with silencing DENND1A.V2 shRNA1 and shRNA2 plasmids significantly inhibited both basal (P<0.05) and 20 μM forskolin (*, P<0.01) stimulated CYP17 mRNA accumulation, as compared to Scrambled plasmid (Fig 7A). Transfection with silencing DENND1A.V2 shRNA plasmids on PCOS theca also significantly inhibited forskolin-stimulated (*, P< 0.05) CYP11A1 mRNA accumulation (Fig 7B). As shown in Fig 7C, co-transfection of -235/+44 of the *CYP17A1* promoter fused to the luciferase gene in a pGL3 plasmid (-235 *CYP17A1*/LUC) with DENND1A.V2 shRNA1 and shRNA2 plasmids resulted in a significant inhibition of forskolin-dependent *CYP17A1* reporter activity in PCOS theca cells, compared to Scrambled shRNA *(P<0.05*). Infection with silencing shRNA DENND1A.V2 lentivirus particles significantly inhibited forskolin-stimulated 17OHP4 (Fig 7D; *, P< 0.001), DHEA (Fig 7E; *, P< 0.001), and progesterone biosynthesis (Fig 7F; *, P< 0.001), as compared to control non-silencing lentivirus. Thus, knock-down of DENND1A.V2 in PCOS theca cells converts the PCOS theca cells to a normal phenotype.

[00016] **Figure 8A-F. Rabbit polyclonal DENND1A.V2 specific IgG significantly reduces androgen biosynthesis and CYP17 and CYP11A1 mRNA PCOS theca cells.** PCOS theca cells were treated with increasing concentrations (0.1 - 3.0 μg/mL) of an affinity purified rabbit polyclonal IgG that was generated against the unique C-terminal 21 amino acid sequence of DENND1A.V2 (i.e., QKSITHFAAKFPTRGWTSSSH) (SEQ ID NO:6), or non-specific IgG, in the absence (C) or presence of 20 μM forskolin (F). In Fig 8A, rabbit polyclonal DENND1A.V2 IgG significantly inhibited forskolin-stimulated DHEA biosynthesis, with an approximate ID_{50} of 0.25 μg/mL, compared to non-specific IgG. Experiments were performed to

examine the effects of 0.5 µg/mL of polyclonal DENND1A.V2 specific IgG or 0.5 µg/mL non specific IgG on CYP17 (Fig 8B) and CYP11A1 mRNA (Fig 8C) accumulation in the absence (C) or presence of 20 µM forskolin (F), demonstrated that DENND1A.V2 specific IgG significantly inhibits CYP17A mRNA accumulation under control (*, P<0.01) and CYP17 mRNA and CYP11A1 mRNA under forskolin-stimulated (**, P<0.01) conditions in PCOS theca cells, while having no effect in normal theca cells. Parallel experiments to examine the effects of 0.5 µg/mL of DENND1A.V2 IgG or 0.5 µg/mL or control IgG on basal and forskolin-stimulated DHEA (Fig 8D), 17OHP4 (Fig 8E), and P4 (Fig 8F) biosynthesis similarly demonstrated that polyclonal DENND1A.V2 specific IgG significantly inhibited forskolin-stimulated DHEA (Fig 8D, *, P< 0.001) and 17OHP4 (Fig 8E, *, P< 0.001), in PCOS theca cells, as compared to control IgG, without affecting normal theca cells.

[00017] **Figure 9A-B. Phage Display Screening of a Human Phage Library with DENND1A.V2 Peptide Identified Two Clones with Identical ScFv Sequences (SEQ ID NOs: 52-53).** To obtain a humanized monoclonal antibody, a freshly made human phage library was used to perform phage display screening with the biotinylated C-terminal DENND1A.V2 21 amino acid peptide (i.e., QKSITHFAAKFPTRGWTSSSH) (SEQ ID NO:6). Two ScFv clones, clones 16 and 79 were identified following ELISA screening (Tables 2-5). Sequence analysis of these clones demonstrated that the nucleotide (Fig 9A) (SEQ ID NO:52) and amino acid (Fig 9B) (SEQ ID NO:53) sequence of clones 16 and 79 were identical. As shown in Fig 9B, the VH sequence comprises amino acid residues 1-116. The linker comprises residues 117-132 and the VL sequence comprises residues 133-240. For both Fig 9A and 9B, the variable region of the heavy chain (VH) is in capitals; the linker is in lower case; the variable region of the light chain (VL) is in underlined, italicized capitals. The complementary determining regions (CDRs) are in boxes (Fig 9B).

[00018] **Figure 10A-D. Sequences of the Heavy and Light Chains of Humanized Monoclonal (Recombinant) Antibodies to DENND1A.V2 Peptide (SEQ ID NOs: 56-59).** The VL and HL sequences of the scFv identified following phage display were cloned into the expression plasmids pIgG1-L and pIgG1-L. The sequence of the of the full length heavy chain of IgG1 (Fig 10A; SEQ ID NO:56) and light chain of IgG1(Fig 10B; SEQ ID NO:57) of humanized monoclonal, or recombinant DENND1.V2 IgG1 were determined. The secretory signal peptide (SP) sequence for both the light and heavy chains is italicized in both Figs 10A-

10B. For the heavy chain in Fig 10A, the variable region of the heavy chain (VH) is upper case (i.e., capitals), and the remainder of the constant region of the heavy chain of human IgG1 is in lower case. For the light chain in Fig 10B, the variable region of the light chain (VL) is italicized, underlined upper case, and the remainder of the constant region of the light chain of human IgG1 is in lower case. The protein sequence of the heavy and light chains are presented in Figs 10C-D. For both the heavy and light chains, the secretory signal peptide (SP) sequence is in italicized capitals, and the CDR are underlined and bracketed. For the heavy chain in Fig 10C (SEQ ID NO:58), the variable region of the heavy chain (VH) is in capitals, the remainder of the constant region of the heavy chain of human IgG1 is in capitals. For the light chain in Fig 10D (SEQ ID NO:59), the variable region of the heavy chain (VL) is in italicized capitals, and the remainder of the constant region of the light chain of human IgG1 is in capitals.

[00019] Figure 11A-C. Human Recombinant DENND1A.V2 specific IgG1 expression and purification. To further assess the humanized monoclonal antibody that was obtained following phage screening, plasmid vectors encoding the heavy and light chains of the recombinant human DENND1A.V2 specific IgG1 were transfected and expressed in 293E cells. The suspension culture was collected 96 hours after transfection. The product was purified by HiTrap rProteinA FF and filtered by 0.2μm. As shown in Fig 11A, the humanized monoclonal antibody, or recombinant DENND1A.V2 specific IgG1 had a calculated concentration of 126μg/ml. Reducing SDS-PAGE analysis demonstrated that the heavy and light chains of the recombinant DENND1A.V2 IgG1 are of the appropriate size (Fig 11B). ELISA results demonstrating specific binding of the human recombinant DENND1A.V2 IgG1 to the 21 amino acid biotinylated DENND1A.V2 sequence (Bio-CH-22) are shown in Fig 11C.

[00020]

Figure 12A-B. Recombinant human DENND1A.V2 specific IgG1 functionally inhibits DHEA biosynthesis in PCOS theca cells in a dose dependent manner. To examine whether recombinant human IgG1 specific to DENND1A.V2 functionally inhibits androgen biosynthesis, PCOS theca cells were treated with increasing concentrations (.01-6 μg/mL) of the human recombinant monoclonal IgG1 specific for DENND1A.V2 (V2 hmAB) or a non-specific IgG1, treated in the absence (C) and presence of 20 μM forskolin (F) for 72h. As shown in Fig 12A, V2 hmAB significantly decreased (*, $P<0.01$) forskolin-stimulated DHEA biosynthesis with an approximate ID_{50} of 1.8 μg/mL, compared to control IgG1. In Fig 12B, both basal and forskolin

(*, P<0.1) stimulated DHEA biosynthesis were inhibited following treatment with V2 hmAB as compared to control IgG1.

[00021] **Figure 13. Effects of recombinant human DENND1A.V2 specific IgG1 on DHEA biosynthesis in normal and PCOS theca cells. Both rabbit polyclonal and human recombinant DENND1A.V2 reduce DHEA biosynthesis in PCOS theca cells.** Experiments performed to compare the effect of 9 μg/mL human recombinant DENND1A.V2 IgG1 (V2 hmAB) and non-specific IgG1 in normal and PCOS theca cells treated in the absence (C; control) and presence of 20 μM forskolin. As shown in Fig 13A, a maximal dose of V2 hmAB significantly (*, P<0.01) inhibited forskolin-stimulated DHEA biosynthesis in PCOS theca cells, while having minimal effect on normal theca cells. A comparison of the effects rabbit DENND1A.V2 polyclonal antibody (V2 polyclonal) versus human recombinant DENND1A.V2 IgG1 (V2 hmAB Recominant) demonstrates that both of these antibodies similarly reduce DHEA biosynthesis as compared to control IgG (Fig 13B).

[00022] **Figure 14. Mouse monoclonal antibody reactivity of selected hybridomas in ELISA to DENND1A peptide.** An ELISA scan of mouse monoclonal antibody supernatants for selected hybridomas was performed. Each hybridoma supernatant (1:10 dilution) was tested for binding reactivity to DENND1A.V2 antigens including free peptide (V2-peptide, QKSITHFAAKFPTRGWTSSSH) (SEQ ID NO:6), labeled V2-KLH-peptide and V2-BSA-peptide, as well as control peptide and BSA. A control peptide to the HPV16 L2 (L2 peptide) sequence (aa17-36) and a monoclonal reactive to this peptide (L216.1A), were used as internal negative control monoclonal antibodies. A second control monoclonal (BSA-1) was reactive to BSA. Several monoclonal antibodies were identified to have specific reactivity to DENND1A.V2 free peptide as well as V2-KLH- and V2-BSA labeled peptide, including P1B2, P1C5, P4F1, P5F9, and P2F5.

[00023] **Figure 15. Inhibitory effects of mouse monoclonal DENND1A.V2 IgG1 on androgen biosynthesis.** Experiments were performed to examine the functional effects of the DENND1A.V2 mouse monoclonal antibodies, P1B2 and P1C5, that were shown to be reactive in the ELISA screen (Fig 14) on androgen biosynthesis in PCOS theca cells. PCOS theca cells were treated with 40 μL of tissue culture supernatant of either P1B2 (mV2.A) and P1C5 (mV2.B) mouse IgG1, or negative control mouse IgG1, in the presence and absence of 20 μM forskolin for 72h. As shown in Fig 15, DENND1A.V2 specific P1B2 and P1C5 mouse IgG1

inhibited forskolin-stimulated DHEA accumulation in PCOS theca cells (*, $P < 0.05$), compared to negative control mouse IgG1.

[00024] **Figure 16A-F. Sequences of the DENND1A.V2 specific mouse monoclonal antibodies P1B2 and P1C5 (SEQ ID NOs:60-65).** The mouse monoclonal DENND1A.V2 specific IgG1, P1B2 heavy chain (Fig 16A) (SEQ ID NO:60), P1B2 light chain (Fig 16B) (SEQ ID NO:61), and the heavy chain of P1C5 (Fig 16E)(SEQ ID NO:64) nucleotide sequences were determined. The DNA sequence of the heavy chain of P1B2 is in capitals, and light chain of P1B2 is in underlined and italicized capitals. For the protein sequences of the heavy chain of P1B2 (Fig 16C) (SEQ ID NO:62) and the heavy chain of P1C5 (Fig 16F) (SEQ ID NO:65) are in capitals, and light chain of P1B2 is italicized (Fig 16D) (SEQ ID NO:63) The CDR residues are underlined and bracketed. The uncertain amino acid sequences are indicated in italics.

DETAILED DESCRIPTION

[00025] Embodiments of the invention relate to a pharmaceutical composition comprising an antibody or an antigen binding fragment thereof, wherein the antibody or the antigen binding fragment thereof specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein. Additional embodiments of the invention relate to a method for prophylaxis and/or therapy of a disorder that is positively correlated with expression of DENND1A.V2 mRNA and/or protein comprising administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical composition comprising an antibody or an antigen binding fragment thereof, wherein the antibody or the antigen binding fragment thereof specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein.

[00026] **The DENND1A.V2 cDNA sequence is:**

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0001 cgcgcgccgg gcacgcgcgc cggcgaccat ggcgttcgcc gggctggagc gagtacatta
0061 acccctggag gcggcggcgg cggcgaggga gcgagcctcg agcgggcggg ccccagcctg
0121 agggaaggga ggaaggggcg gggagagcgc cagagggagg ccggtcggcc gcgggcgggc
0181 gggcagcgca gcgccgagcg gggcccgcgg gcccatgagg aggcctgggg accatgggct
0241 ccaggatcaa gcagaatcca gagaccacat ttgaagtata tgttgaagtg gcctatccca
0301 ggacaggtgg cactctttca gatcctgagg tgcagaggca attcccggag gactacagtg
0361 accaggaagt tctacagact ttgaccaagt tttgtttccc cttctatgtg gacagcctca
0421 cagttagcca agttggccag aacttcacat tcgtgctcac tgacattgac agcaaacaga
0481 gattcgggtt ctgccgctta tcttcaggag cgaagagctg cttctgtatc ttaagctatc
0541 tcccctggtt cgaggtattt tataagctgc ttaacatcct ggcagattac acgacaaaaa
0601 gacaggaaaa tcagtggaat gagcttcttg aaactctgca caaacttccc atccctgacc
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0661 caggagtgtc tgtccatctc agcgtgcatt cttattttac tgtgcctgat accagagaac
0721 ttcccagcat acctgagaat agaaatctga cagaatattt tgtggctgtg gatgttaaca
0781 acatgttgca tctgtacgcc agtatgctgt acgaacgccg gatactcatc atttgcagca
0841 aactcagcac tctgactgcc tgcatccacg ggtctgcggc gatgctctac cccatgtact
0901 ggcagcacgt gtacatcccc gtgctgccgc cgcatctgct ggactactgc tgtgctccca
0961 tgccctacct cataggaatc catttaagtt taatggagaa agtcagaaac atggccctgg
1021 atgatgtcgt gatcctgaat gtggacacca acaccctgga aacccccttc gatgacctcc
1081 agagcctccc aaacgacgtg atctcttccc tgaagaacag gctgaaaaag gtctccacaa
1141 ccactgggga tggtgtggcc agagcgttcc tcaaggccca ggctgctttc ttcggtagct
1201 accgaaacgc tctgaaaatc gagccggagg agccgatcac tttctgtgag gaagccttcg
1261 tgtcccacta ccgctccgga gccatgaggc agttcctgca gaacgccaca cagctgcagc
1321 tcttcaagca gtttattgat ggtcgattag atcttctcaa ttccggcgaa ggtttcagtg
1381 atgtttttga agaggaaatc aacatgggcg agtacgctgg cagtgacaaa ctgtaccatc
1441 agtggctctc cactgtccgg aaaggaagtg gagcaattct gaatactgta aagaccaaag
1501 caaatccggc catgaagact gtctacaagt tcgcaaaaga tcatgcaaaa atgggaataa
1561 aagaggtgaa aaaccgcttg aagcaaaagg acattgccga gaatggctgc gcccccaccc
1621 cagaagagca gctgccaaag actgcaccgt ccccactggt ggaggccaag gaccccaagc
1681 tccgagaaga ccggcggcca atcacagtcc actttggaca ggtgcgccca cctcgtccac
1741 atgttgttaa gagaccaaag agcaacatcg cagtggaagg ccggaggacg tctgtgccga
1801 gccctgagca aaacaccatt gcaacaccag ctacactcca catcctacag aaaagcatta
1861 cccattttgc ggccaagttc ccgacgagag gctggacctc ttcatcacat tgacttacgc
1921 cgttgctttt ccagactggg cagaggggct gacttcgcag tgtgtgccaa agagccggtg
1981 tctgataatc ccattttcct gcttatcacc tgaactgtgt cagtatcact tttagttttg
2041 ttggttggtt ggtttgttgt ttgtttaata tgccctgttt tctacttctg ttggaaaata
2101 tttggggttg aaataaacca gtgggagcat ggaaaaaaaa aaaaaaaaaa aaaaaaaaaa
2161 aaaaaa (SEQ ID NO:1)

[00027] The DENND1A.V2 amino acid sequence is:

MGSRIKQNPETTFEVYVEVAYPRTGGTLSDPEVQRQFPEDYSDQEVLQTLTKFCFPFYVDSLTVSQVGQNFTFVLTD
IDSKQRFGFCRLSSGAKSCFCILSYLPWFEVFYKLLNILADYTTKRQENQWNELLETLHKLPIPDPGVSVHLSVHSY
FTVPDTRELPSIPENRNLTEYFVAVDVNNMLHLYASMLYERRILIICSKLSTLTACIHGSAAMLYPMYWQHVYIPVL
PPHLLDYCCAPMPYLIGIHLSLMEKVRNMALDDVVILNVDTNTLETPFDDLQSLPNDVISSLKNRLKKVSTTTGDGV
ARAFLKAQAAFFGSYRNALKIEPEEPITFCEEAFVSHYRSGAMRQFLQNATQLQLFKQFIDGRLDLLNSGEGFSDVF
EEEINMGEYAGSDKLYHQWLSTVRKGSGAILNTVKTKANPAMKTVYKFAKDHAKMGIKEVKNRLKQKDIAENGCAPT
PEEQLPKTAPSPLVEAKDPKLREDRRPITVHFGQVRPPRPHVVKRPKSNIAVEGRRTSVPSPEQNTIATPATLHILQ
KSITHFAAKFPTRGWTSSSH (SEQ ID NO:2)

[00028] The DENND1A.V1 cDNA sequence is:

0001 cgcgcgccgg gcacgcgcgc cggcgaccat ggcgttcgcc gggctggagc gagtacatta
0061 acccctggag gcggcggcgg cggcgaggga gcgagcctcg agcgggcggg ccccagcctg
0121 agggaaggga ggaaggggcg gggagagcgc cagagggagg ccggtcggcc gcgggcgggc
0181 gggcagcgca gcgccgagcg gggcccgcgg gcccatgagg aggcctgggg accatggct
0241 ccaggatcaa gcagaatcca gagaccacat ttgaagtata tgttgaagtg gcctatccca
0301 ggacaggtgg cactctttca gatcctgagg tgcagaggca attcccggag gactacagtg
0361 accaggaagt tctacagact ttgaccaagt tttgtttccc cttctatgtg gacagcctca
0421 cagttagcca agttggccag aacttcacat tcgtgctcac tgacattgac agcaaacaga
0481 gattcgggtt ctgccgctta tcttcaggag cgaagagctg cttctgtatc ttaagctatc
0541 tcccctggtt cgaggtattt tataagctgc ttaacatcct ggcagattac acgacaaaaa

0601 gacaggaaaa tcagtggaat gagcttcttg aaactctgca caaacttccc atccctgacc
0661 caggagtgtc tgtccatctc agcgtgcatt cttattttac tgtgcctgat accagagaac
0721 ttcccagcat acctgagaat agaaatctga cagaatattt tgtggctgtg gatgttaaca
0781 acatgttgca tctgtacgcc agtatgctgt acgaacgccg gatactcatc atttgcagca
0841 aactcagcac tctgactgcc tgcatccacg ggtctgcggc gatgctctac cccatgtact
0901 ggcagcacgt gtacatcccc gtgctgccgc cgcatctgct ggactactgc tgtgctccca
0961 tgccctacct cataggaatc catttaagtt taatggagaa agtcagaaac atggccctgg
1021 atgatgtcgt gatcctgaat gtggacacca acaccctgga aacccccttc gatgacctcc
1081 agagcctccc aaacgacgtg atctcttccc tgaagaacag gctgaaaaag gtctccacaa
1141 ccactgggga tggtgtggcc agagcgttcc tcaaggccca ggctgctttc ttcggtagct
1201 accgaaacgc tctgaaaatc gagccggagg agccgatcac tttctgtgag gaagccttcg
1261 tgtcccacta ccgctccgga gccatgaggc agttcctgca gaacgccaca cagctgcagc
1321 tcttcaagca gtttattgat ggtcgattag atcttctcaa ttccggcgaa ggtttcagtg
1381 atgtttttga agaggaaatc aacatgggcg agtacgctgg cagtgacaaa ctgtaccatc
1441 agtggctctc cactgtccgg aaaggaagtg gagcaattct gaatactgta aagaccaaag
1501 caaatccggc catgaagact gtctacaagt tcgcaaaaga tcatgcaaaa atgggaataa
1561 aagaggtgaa aaaccgcttg aagcaaaagg acattgccga gaatggctgc gcccccaccc
1621 cagaagagca gctgccaaag actgcaccgt ccccactggt ggaggccaag gaccccaagc
1681 tccgagaaga ccggcggcca atcacagtcc actttggaca ggtgcgccca cctcgtccac
1741 atgttgttaa gagaccaaag agcaacatcg cagtggaagg ccggaggacg tctgtgccga
1801 gccctgagca gccgcagccg tatcggacac tcagggagtc agacagcgcg gaaggcgacg
1861 aggcagagag tccagagcag caagtgcgga agtccacagg ccctgtccca gctccccctg
1921 accgggctgc cagcatcgac cttctggaag acgtcttcag caacctggac atggaggccg
1981 cactgcagcc actgggccag gccaagagct tagaggacct tcgtgccccc aaagacctga
2041 gggagcagcc agggaccttt gactatcaga ggctggatct gggcgggagt gagaggagcc
2101 gcggggtgac agtggccttg aagcttaccc acccgtacaa caagctctgg agcctgggcc
2161 aggacgacat ggccatcccc agcaagcccc cagctgcctc ccctgagaag ccctcggccc
2221 tgctcgggaa ctccctggcc ctgcctcgaa ggccccagaa ccgggacagc atcctgaacc
2281 ccagtgacaa ggaggaggtg cccaccccta ctctgggcag catcaccatc ccccggcccc
2341 aaggcaggaa gaccccagag ctgggcatcg tgcctccacc gcccattccc cgcccggcca
2401 agctccaggc tgccggcgcc gcacttggtg acgtctcaga gcggctgcag acggatcggg
2461 acaggcgagc tgccctgagt ccagggctcc tgcctggtgt tgtcccccaa ggccccactg
2521 aactgctcca gccgctcagc cctggccccg ggctgcaggg cacgagcagt gacgccctgc
2581 tcgccctcct ggacccgctc agcacagcct ggtcaggcag caccctcccg tcacgccccg
2641 ccaccccgaa tgtagccacc ccattcaccc cccaattcag cttccccccct gcagggacac
2701 ccaccccatt cccacagcca ccactcaacc cctttgtccc atccatgcca gcagccccac
2761 ccaccctgcc cctggtctcc acaccagccg ggcctttcgg ggcccctcca gcttccctgg
2821 ggccggcttt tgcgtccggc ctcctgctgt ccagtgctgg cttctgtgcc cctcacaggt
2881 ctcagcccaa cctctccgcc ctctccatgc ccaacctctt tggccagatg cccatgggca
2941 cccacacgag ccccctacag ccgctgggtc cccagcagt tgccccgtcg aggatccgaa
3001 cgttgcccct ggcccgctca agtgccaggg ctgctgagac caagcagggg ctggccctga
3061 ggcctggaga cccccccgctt ctgcctccca ggccccctca aggcctggag ccaacactgc
3121 agccctctgc tcctcaacag gccagagacc cctttgagga tttgttacag aaaaccaagc
3181 aagacgtgag cccgagtccg gccctggccc cggccccaga ctcggtggag cagctcagga
3241 agcagtggga gaccttcgag tgagccgggc cctgagggtg ggggatgcac cgaggcccga
3301 gggtccgtcc actgctgcgg ttccgaggct cccccgccac tctctctctg cccaggttct
3361 gctggtggga agggatggga cccctctctg ctgccccctc ctcccctcca cactgcccat
3421 ctctgatgtc tggccctggg gaatggcacc agttccagcc tgggaatcaa cccagttcct
3481 gagtgcccat cccacccccgc ggttgcctct cctcggcacc cttgattggg ttttgcacta
3541 aagaggtcag ctgggccaat gatattgctc cagaccgagt cctacccacc ttcccccgga
3601 agtgtcccaa gaggctccga aggcctcccc tccgagccca gctctcctgt ctcctccaca
3661 gccaggccct gcacgcccac ctcctcggac acaggtgaca gggttaccct ccagtttgag
3721 ctcatctgca cgagacacag gtagcttggg gttgaagtta ggactcctcc tgggctggag
3781 gatttacctg gtggggcact tccagactgt ttctagcaat atacacacac gttctttcct

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3841 gtgtcttcac cccaaaactt cagttgattc tgacctggga ggatctgggg accagggggt
3901 cttgggctgc cttgtgatac acagccccag ccaccctgca cgggggctgc gagcaccagc
3961 aactttgatt tatagaagga aaatggaaac ccccatctga gtattttggg aggagccccc
4021 agccctcatc cagctctggc acgctgatac ctccaggtac tcccctcact gtcaaagctg
4081 gggctcagcc tcttgtcatc tggagctttg tgggcaaagc tgagaagctg caacccagat
4141 ttcaacccaa aaaggtcaag ctgaatgcct cagactgatg tggaaggcag ctggccttcc
4201 tgggttggaa cgaggcagtg gccctgagcc ccttctccag ggccaggtag aaaggacaaa
4261 cttggtctct gcctcgggga agcaggagga gggctagaag ccagtccctc cccacctgcc
4321 cagagctcca ggccagcaca gaaattcctg aggccaacgt caccaaagtt agattgaatg
4381 tttattatct ttcttttcc tttttacctt attgatttga tgaatcttga aatggattca
4441 tttccataaa ccaagttaaa gtatggcccg accatttaag aaaacaacca tctgagacac
4501 gcaggaaatt gtgagcattt cgacccgagc tctcatttcc tatttgtgaa gggtcagaca
4561 cagtctaccc aggggtgtct gggggacaag gggtctctg gagatgtcac ccagggagcc
4621 ccctctatgt ctgagaggct gccactgctg cacatgctca gtgaggcttg gcggccatcc
4681 tggcacatgg ctcttcctgg gtcaaccgtg acctgtctgg ctcaggaatg ggctctggct
4741 gctgggggag ccgtgtcact cctgggccat gggggcacct cctgggcact taggtgtttc
4801 agcatagatt ccagtttcgc accctgggca gacccccagg ccccatccgg gatagggcag
4861 aggaggtgct ggcggcccca gggaaggagg gtgtgtaccc caaggccccc tggctgtgct
4921 gaggggctgg ggtgagcgct ccatgttcac atgagcactg ctgcctcttc acttgtggga
4981 ctttttgcaa acccaaggat gaactttgtg tgcattcaat aaaatcatct tggggaagag
5041 g
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(SEQ ID NO:3)

[00029] The DENND1A.V1 amino acid sequence is:

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MGSRIKQNPETTFEVYVEVAYPRTGGTLSDPEVQRQFPEDYSDQEVLQTLTKFCFPFYVDSLTVSQVGQNFTFVLTD
IDSKQRFGFCRLSSGAKSCFCILSYLPWFEVFYKLLNILADYTTKRQENQWNELLETLHKLPIPDPGVSVHLSVHSY
FTVPDTRELPSIPENRNLTEYFVAVDVNNMLHLYASMLYERRILIICSKLSTLTACIHGSAAMLYPMYWQHVYIPVL
PPHLLDYCCAPMPYLIGIHLSLMEKVRNMALDDVVILNVDTNTLETPFDDLQSLPNDVISSLKNRLKKVSTTTGDGV
ARAFLKAQAAFFGSYRNALKIEPEEPITFCEEAFVSHYRSGAMRQFLQNATQLQLFKQFIDGRLDLLNSGEGFSDVF
EEEINMGEYAGSDKLYHQWLSTVRKGSGAILNTVKTKANPAMKTVYKFAKDHAKMGIKEVKNRLKQKDIAENGCAPT
PEEQLPKTAPSPLVEAKDPKLREDRRPITVHFGQVRPPRPHVVKRPKSNIAVEGRRTSVPSPEQPQPYRTLRESDSA
EGDEAESPEQQVRKSTGPVPAPPDRAASIDLLEDVFSNLDMEAALQPLGQAKSLEDLRAPKDLREQPGTFDYQRLDL
GGSERSRGVTVALKLTHPYNKLWSLGQDDMAIPSKPPAASPEKPSALLGNSLALPRRPQNRDSILNPSDKEEVPTPT
LGSITIPRPQGRKTPELGIVPPPPIPRPAKLQAAGAALGDVSERLQTDRDRRAALSPGLLPGVVPQGPTELLQPLSP
GPGAAGTSSDALLALLDPLSTAWSGSTLPSRPATPNVATPFTPQFSFPPAGTPTPFPQPPLNPFVPSMPAAPPTLPL
VSTPAGPFGAPPASLGPAFASGLLLSSAGFCAPHRSQPNLSALSMPNLFGQMPMGTHTSPLQPLGPPAVAPSRIRTL
PLARSSARAAETKQGLALRPGDPPLLPPRPPQGLEPTLQPSAPQQARDPFEDLLQKTKQDVSPSPALAPAPDSVEQL
RKQWETFE
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(SEQ ID NO:4)

[00030] For each cDNA sequence presented herein, the invention includes the mRNA equivalent of the cDNA, meaning that the invention includes each cDNA sequence wherein each T is replaced by U.

[00031] In one aspect, the present disclosure comprises one or more isolated and/or recombinantly or otherwise synthesized (e.g. chemically synthesized) binding partners which specifically recognize the DENND1A.V2 protein, but do not specifically recognize DENND1A.V1 protein.

[00032] Such binding partners include antibodies and antigen binding fragments thereof that can specifically bind to the unique C-terminus of the DENND1A.V2 protein, such as Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fd fragments, Fv fragments, scFv fragments, aptamers, diabodies and combinations thereof. Single-chain Fv or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. The antibody and antigen binding fragments thereof are directed to one or more epitopes in the unique 33 amino acid C-terminus of the DENND1A.V2 protein. The 33 amino acid DENND1A.V2 sequence that is unique between DENND1A Variants 1 and 2 is: NTIATPATLHILQKSITHFAAKFPTRGWTSSSH (SEQ ID NO:5).

[00033] This disclosure includes a demonstration of making antibodies directed to a segment of this unique DENND1A.V2 C-terminal amino acid sequence. In this regard, a DENND1A.V2 polyclonal antibody (rabbit) targeted to the following unique 21 amino acid sequence segment: QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) was generated. This sequence was selected based on an analysis that predicted it to be optimally antigenic, and unlikely to result in production of antibodies that would have non-specific cross-reactivity with other proteins.

[00034] In this disclosure, it is demonstrated that antibody targeting of the 21 amino acid C-terminus of the DENND1A.V2 protein significantly reduces androgen biosynthesis and CYP17 and CYP11A1 mRNA PCOS theca cells (e.g., see Fig 8A-B).

[00035] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained

from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

[00036] Antibodies and methods for preparation of antibodies are well-known in the art. Details of methods of antibody generation and screening of generated antibodies for substantially specific binding to an antigen are described in standard references such as E.Harlow and D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988; F. Breitling and S. Dübel, Recombinant Antibodies, John Wiley & Sons, New York, 1999; H. Zola, Monoclonal Antibodies: Preparation and Use of Monoclonal Antibodies and Engineered Antibody Derivatives, Basics: From Background to Bench, BIOS Scientific Publishers, 2000; and B.K.C. Lo, Antibody Engineering: Methods and Protocols, Methods in Molecular Biology, Humana Press, 2003.

[00037] In one illustrative approach, a monoclonal antibody can be made by standard approaches using the polypeptide NTIATPATLHILQKSITHFAAKFPTRGWTSSSH (SEQ ID NO:5) that is located at the DENND1A.V2 C-terminus, or any immunogenic fragment of it. In an embodiment, a monoclonal antibody is made to a 21 amino acid sequence segment of SEQ ID NO: 5, consisting of the sequence QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) or to one or more determinants within SEQ ID NO:6.

[00038] Antibodies of the present disclosure can recognize either a conformational epitope(s) and/or a linear epitope(s) present in the NTIATPATLHILQKSITHFAAKFPTRG WTSSSH (SEQ ID NO:5) sequence or QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6).

[00039] In embodiments, the antibodies and antigen binding fragments specifically recognize at least one epitope present in at least about 4 contiguous amino acids of the 33 amino acid DENND1A.V2 C-terminal sequence, beginning and ending at any position within the 33 amino acid sequence. In embodiments, the epitope has at least about 4, 5, 6, or 7 contiguous amino acids present in the 33 amino acid DENND1A.V2 C-terminal sequence (SEQ ID NO: 5). The epitope can also be defined by longer sequences, for example up to about 12 amino acids (e.g. 8, 9, 10, 11, or 12 amino acids). Thus, the epitope can comprise or consist of up to any about 12 amino acid segment of the 33 amino acid DENND1A.V2 C-terminal sequence, or a longer segment. In embodiments, the antibody or antigen binding fragment is specific for an epitope that is partially or fully present in any of the following exemplary segments of the 33 amino acid DENND1A.V2 C-terminal sequence: NTIATPA (SEQ ID NO:7); TIATPAT (SEQ

ID NO:8); IATPATL (SEQ ID NO:9); ATPATLH (SEQ ID NO:10); TPATLHI (SEQ ID NO:11); PATLHIL (SEQ ID NO:12); ATLHILQ (SEQ ID NO:13); TLHILQK (SEQ ID NO:14); LHILQKS (SEQ ID NO:15); HILQKSI (SEQ ID NO:16); ILQKSIT (SEQ ID NO:17); LQKSITH (SEQ ID NO:18); QKSITHF (SEQ ID NO:19); KSITHFA (SEQ ID NO:20); SITHFAA (SEQ ID NO:21); ITHFAAK (SEQ ID NO:22); THFAAKF (SEQ ID NO:23); HFAAKFP (SEQ ID NO:24); FAAKFPT (SEQ ID NO:25); AAKFPTR (SEQ ID NO:26); AKFPTRG (SEQ ID NO:27); KFPTRGW (SEQ ID NO:28); FPTRGWT (SEQ ID NO:29); PTRGWTS (SEQ ID NO:30); TRGWTSS (SEQ ID NO:31); RGWTSSS (SEQ ID NO:32); GWTSSSH (SEQ ID NO:33), and combinations thereof. In embodiments, the antibodies of this disclosure specifically recognize an epitope present in at least about 4 contiguous amino acids of the 21 amino acid sequence: QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) (e.g. segments comprising about 4, 5, 6, 7, 8, 9, 10, 11 or 12 consecutive amino acids, which are included in or include the group of 7 amino acid sequences disclosed above. For example, starting from SEQ ID NO:7, the following 4 contiguous amino acids may comprise an epitope that is recognized by the antibody: NTIA (SEQ ID NO:66), TIAT (SEQ ID NO:67), IATP (SEQ ID NO:68), and ATPA (SEQ ID NO:69). Additionally, these epitopes may be part of longer sequences. For example, starting from SEQ ID NO:66 the sequence XXNTIAX (SEQ ID NO:70) may comprise an epitope that is recognized by the antibody wherein X is any amino acid as described herein. In embodiments, the antibodies recognize an epitope with a sequence with at least about 65, 70, 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity to a sequence partially or fully present in SEQ ID NO:5. The disclosure includes structures (e.g. binding molecules such as antibodies or fragments thereof as described herein) that have at least one paratope that recognizes at least one of the foregoing amino acid segments, or at least two paratopes that recognize at least one of the foregoing segments.

[00040] An antibody or antigen-binding fragment of the invention specifically recognizes or binds a target epitope when it displays no more than 5% binding to other epitopes, and preferably displays no measurable binding to other epitopes, for example when measured by an enzyme-linked immunosorbent assay. An antibody or antigen-binding fragment does not specifically recognize or bind a target epitope if it displays more than 5% binding to other epitopes. Antibodies or antigen-binding fragments that bind non-specifically may still bind

selectively, for example, an antibody may bind the target epitope as well as display non-specific binding to a few other epitopes.

[00041] Antibodies may be produced by various methods known in the art such as chemical peptide synthesis. In one approach, to make a monoclonal antibody, a DENND1A.V2 C-terminal polypeptide described herein is introduced into a laboratory animal, such as a mouse, over a series of administrations over a period of time spanning several weeks. Typically, splenocytes are isolated from the mouse spleen, and isolated B cells are obtained and fused with myeloma cells which have been immortalized using any suitable approach, such as electrofusion. The myeloma cells characteristically lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene and as a result are sensitive to HAT medium (hypoxanthine-aminopterin-thymidine medium). The fusions are generally exposed to the HAT medium for a period of time, such as from 10 to 14 days, during which a compound such as aminopterin is used to inhibit nucleotide synthesis, resulting in death of unfused cells and survival of B cell-myeloma hybrids (hybridomas) which have been immortalized and which produce antibodies. The cells are diluted to isolate single hybridomas in single wells of, for instance, a multi-well plate. The hybridomas are then screened to identify those that produce antibodies that specifically recognize the DENND1A.V2 C-terminus, and thus do not cross-react with DENND1A.V1 protein. Once suitable hybridomas are isolated the DNA encoding the secreted immunoglobulin (Ig) can be sequenced, and thus the amino acid sequence of the Ig can be determined. The complementarity determining regions (CDRs) of the Ig heavy and light chains can be determined and used to make synthetic versions of the antibodies made by the hybridomas, or to make antigen binding moieties as further described herein. Alternatively, the cell that produces the antibody can be cloned to produce identical daughter clones which will provide an ongoing source of monoclonal antibodies. When large amounts of antiserum is desired, larger animals, for example goat or sheep, may be used as the host species for antibody production.

[00042] Any antibody produced by a non-human mammal derived hybridoma can be modified to provide a chimeric, or partially or fully humanized form; and the present disclosure includes such modifications. In general, "humanized" forms of non-human (e.g., mice) antibodies are chimeric antibodies that contain a minimal sequence derived from the non-human antibody. Humanized antibodies are essentially human immunoglobulins (also called the "recipient" antibody) in which residues from a hypervariable region of the recipient are replaced

by residues from a hypervariable region of a non-human species (also called a "donor" antibody) such as mouse, rat, rabbit or non-human primate having the desired antibody specificity, affinity, and capability. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also can comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., [27]; Riechmann et al., [28]; and Presta, [29].

[00043] Methods for humanizing non-human antibodies are well known in the art. Humanization of an antibody produced according to the present disclosure can be essentially performed following the method of Winter and co-workers by substituting mouse CDR sequences for the corresponding sequences of a human antibody, Jones et al [27]; Riechmann et al., [28]; and Verhoeyen et al., [30].

[00044] In another embodiment, the disclosure includes an antigen-binding or variable region fragment of an antibody described herein. Examples of suitable antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies. However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from antibody phage libraries as described below. Alternatively, Fab'-SH fragments can be directly recovered from bacterial expression systems and chemically coupled to form F(ab')2 fragments.

[00045] In one embodiment, a library of proteins that have antigen binding regions can be screened to identify candidates that can be modified for therapeutic purposes according to this disclosure. For example, a phage display or other antibody library can be screened against a DENND1A.V2 C-terminal polypeptide described herein, and DENND1A.V2 C-terminal specific binding proteins can be identified. The CDR sequences of the light and heavy chains encoded by the phage DNA, or any other DNA that encodes the antigen binding regions, can be determined

using techniques known to those skilled in the art. The CDR sequences can then be cloned into expression vectors to produce DENND1A.V2 C-terminal specific antibodies or DENND1A.V2 C-terminal specific fragments thereof as described above. Thus, in embodiments, the DENND1A.V2 C-terminal specific antibodies or DENND1A.V2 C-terminal specific fragments thereof as described above will be distinct from polypeptides in the library. In an embodiment, the DENND1A.V2 C-terminal specific antibodies or DENND1A.V2 C-terminal specific fragments thereof do not contain any phage/phagemid protein, including but not necessarily limited to bacteriophage coat protein(s). Newer approaches for generating antibodies or antigen binding fragments such as the non-limiting example of camel nanobodies are contemplated herein.

[00046] The invention also provides isolated nucleic acids encoding the anti-DENND1A.V2 antibodies, vectors and host cells comprising the nucleic acids, and recombinant techniques for the production of the antibodies. An isolated nucleic acid may be operably linked to a heterologous promoter. The present invention further provides isolated nucleic acid molecules and their complements that contain genetic sequences (genes) which encode an amino acid sequence with at least about 65, 70, 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity, with the protein/polypeptide sequences disclosed herein. Exemplary encoding nucleotide sequences are provided, but those of skill in the art will recognize that, due to the redundancy of the genetic code, other nucleotide sequences may also encode the same protein/polypeptide. For recombinant production of the antibody, the nucleic acid encoding the antibody is isolated and inserted into a replicable vector for further cloning (e.g. for amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (for example, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available and known in the art, for example pIgG1-H and PIgG1-L, etc. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[00047] The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not

otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Exemplary expression vectors include but are not limited to CMV or β-actin driven plasmids (i.e, pcDNA, pCM6, pDRIVE-β-act, etc).

[00048] Amino acid sequence modification(s) of the anti-DENND1A.V2 antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the anti-DENND1A.V2 antibody are prepared by introducing appropriate nucleotide changes into the anti-DENND1A.V2 antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-DENND1A.V2 antibody. Substitutions may be conservative or non-conservative. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processing of the anti-DENND1A.V2 antibody, such as changing the number or position of glycosylation sites. In general, the amino acid sequences of such variants are at least about 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to the sequence from which they are derived, or, for fragments, to a contiguous portion of a sequence of the same.

[00049] A useful method for identification of certain residues or regions of the anti-DENND1A.V2 antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis". Here, a residue or group of target residues are identified (e g , charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with DENND1A antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, alanine scanning or random mutagenesis is conducted at the target codon or region and the expressed the anti-DENND1A.V2 antibody variants are screened for the desired activity.

[00050] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as

well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-DENND1A.V2 antibody with an N terminal methionyl. Other insertional variants of the anti-DENND1A.V2 antibody molecule include the fusion to the N- or C-terminus of the anti-DENND1A.V2 antibody to an enzyme or a polypeptide which increases the serum half-life of the antibody

[00051] Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-DENND1A.V2 antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated.

[00052] Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

[00053] Any cysteine residue not involved in maintaining the proper conformation of the anti-DENND1A.V2 antibody may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. For example, the two cysteines in the V_H of SEQ ID NO:58 can be replaced with serine as follows: EVQLLESGGGLVQPGGSLRLS**S**AASGFTFSSYAMSWVRQAPGKGLEWVSIIGTDGDDTN YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY**S**AKAEASFDYWGQGTLVTVSS (SEQ ID NO:71). Conversely, residues capable of forming disulfide bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

[00054] A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (for example, a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (for example, 6-7 sites) are mutated to generate all possible amino substitutions at each site. The phage-displayed variants are then screened for their biological activity (for example, binding affinity, appropriate effects on

cellular function). In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human DENND1A.V2. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development. Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody By "altering" we mean deleting one or more residues capable of being glycosylated, or substituting one or more residues that are typically glycosylated or residues that are not susceptible to glycosylation and/or adding one or more amino acids that are glycosylation sites. For example, a N-linked glycosylation motif may be added in the V_H of SEQ ID NO:58 as follows:

EVQLLESGGGLVQPGGSLRLSSAASGFTFSSYAMSWVRQAPGKGLEWVSIIGTDGDDTN
Y**T**DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYSAKAEASFDYWGQGTLVTVSS
(SEQ ID NO:72).

[00055] Embodiments of the invention relate to the treatment of DENND1A.V2 related disorders. The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in whom the disorder is to be prevented. Hence, the mammal to be treated herein may have been diagnosed as having the disorder or may be predisposed or susceptible (e.g. having a genetic predisposition) to the disorder.

[00056] The term "mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

[00057] In some embodiments, the antibodies and/or antigen binding fragments of the invention are provided in a pharmaceutical formulation, which can contain components such as pharmaceutically acceptable carriers, excipients, stabilizers, etc. The antibodies and/or antigen binding fragments may also be combined with other drugs or therapies for the treatment of disorders correlated with expression of DENND1A.V2 mRNA or protein. For example, a

monoclonal antibody against DENND1A.V2 may be administered for the treatment of PCOS in combination with hormonal therapy.

[00058] PCOS, type II diabetes associated with PCOS, and other disorders of females and males that is the direct result of expression of DENND1A.V2 which results in excess steroid biosynthesis are examples of a disease or disorder positively correlated with expression of DENND1A.V2 mRNA or protein. Affected tissues include, but are not limited to: ovarian, vascular smooth muscle, skeletal muscle, adipose tissue, and the endometrium.

[00059] The term "therapeutically effective amount" refers to an amount of a drug or pharmaceutical composition effective to treat a disease or disorder in a subject. In the case of PCOS or another DENND1A.V2 related disorder, the therapeutically effective amount of the antibody may reduce and/or prevent to some extent one or more of the symptoms associated with the disorder. For example, the amount of measurable testosterone (total and free), androstenedione, and dehydroepiandrosterone sulfate (DHEAS) in a biological sample obtained from a subject being treated as described herein (e.g. a blood sample) may decrease, e.g. at least about 10, 20, 30, 40, 50, 60, 70, 80 or 90% or more; and/or other symptoms may be ameliorated, e.g. ovulatory cycles will return, ovarian morphology may normalize, including disappearance of multiple follicular cysts, and hyperinsulinemia and insulin resistance may be diminished.

[00060] In further embodiments, the antibodies or antigen binding fragments thereof may be administered by any suitable means, including, but not limited to; subcutaneous, parenteral, vaginally, intraperitoneal, intrapulmonary, and intranasal. Parenteral infusions include intravenous, intramuscular, intraarterial, intraperitoneal, intralymphatic or subcutaneous administration. In addition, the monoclonal antibodies and/or antigen binding fragments thereof may be administered by pulse infusion, e.g., with declining doses. Methods of oral administration are also contemplated. Slow-release formulations may be used with modifications such as pegylation to extend the half-life of the pharmaceutical composition.

[00061] For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, the severity and course of the disease, whether the antibody is administered for preventive and/or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (for

example, 0.1- 20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The preferred dosage of the antibody will be in the range from about 0.5 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2 mg/kg, 4 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, for example, every week or every three weeks. An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-DENND1A.V2 antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays. Aside from administration of the antibody protein to the patient, the present application contemplates administration of the antibody by gene therapy. Such administration of nucleic acid encoding the antibody is encompassed by the expression "administering a therapeutically effective amount of an antibody".

[00062] Embodiments of the invention include polynucleotides encoding the antibodies and antigen binding fragments thereof, expression vectors comprising those polynucleotides, *in vitro* cell cultures wherein the cells comprise the expression vectors and express the antibodies or the antigen binding fragments thereof, and methods of using such expression vectors and cell cultures for making the antibodies and antigen binding fragments thereof. Other embodiments include RNAi, shRNA or other nucleotide containing compositions for the treatment of a disorder that is positively correlated with expression of DENND1A.V2.

[00063] There are two major approaches to delivering the nucleic acid (optionally contained in a vector) into the patient's cells, *in vivo* and *ex vivo*. For *in vivo* delivery the nucleic acid is injected directly into the patient, e.g. into the bloodstream or at the site where the antibody is required. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are re-implanted into the patient. There are a variety of techniques available for introducing nucleic acids into viable cells.

The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro* or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for *ex vivo* delivery of the gene is a retrovirus.

[00064] Exemplary *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Choi, for example). In some situations, it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein of the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake; for example capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life.

[00065] In some embodiments, the targeted cells are theca cells and contacting the theca cells with the antibodies or antigen binding fragments described herein decreases androgen and/or progesterone biosynthesis in the theca cells and/or decreases *CYP17A1* and/or *CYP11A1* gene expression, as well as CYP17 and CYP11A1 mRNA and/or protein in the cells. These decreases reduce or prevent symptoms associated with overexpression of *CYP17A1* and/or *CYP11A1* gene regulation and CYP17 and/or CYP11A1 mRNA and/or protein; for example, excess androgen production. Exemplary symptoms of excess androgen production which may be lessened or resolved include but are not limited to: anovulation associated with hyperandrogenemia, hirsutism, ovarian morphology, infertility. In some embodiments, the antibodies or antigen binding fragments described herein reduce or prevent other phenotypes associated with PCOS, for example abnormal insulin signaling, insulin resistance in adipose, skeletal muscle, and endometrial tissue, abnormal FSH signaling in granulosa cells, and combinations thereof.

[00066] Embodiments of the invention also provide methods for altering signaling by cell surface receptors in cells having increased DENND1AV.2 expression as compared to control cells, The methods comprise a step of contacting such cells with an antibody or antigen binding

fragment thereof as described herein, wherein the antibody or antigen binding fragment specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein.

[00067] Articles of manufacture containing materials useful for the treatment of the disorders described herein are also provided. The articles of manufacture comprise a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an anti-DENND1A.V2 antibody or antibody fragment as described herein. The label or package insert indicates that the composition is used for treating the condition of choice and may provide instructions for use of the composition.

[00068] Anti-DENND1A.V2 antibodies are useful in diagnostic assays for DENND1A.V2 protein, for example, detecting its expression in specific cells, tissues, or serum. Aspects of the invention provide a method for diagnosis of a disorder that is positively correlated with expression of DENND1A.V2 mRNA and/or protein in a subject comprising contacting a biological sample from said subject with one or more antibodies comprising the amino acid sequence represented by SEQ ID NOs:53, 58, 59, 62, 63 or 65 and comparing the amount of DENND1A.V2 detected to a reference negative control. The disorder may be PCOS. A reference value may be obtained from samples from healthy, normal patients without the disease. If the measured value is greater than the normal reference value, then it can be concluded that the subject has the disease, and if the measured value is the same or less than the normal reference value, then it can be concluded that the subject does not have the disease. In certain embodiments, determining a statistically significant increase in DENND1A Variant 2 mRNA of at least 1.5 fold, and 2-3 fold of DENND1A Variant 2 protein in a sample as compared to a reference is a diagnosis of PCOS or aids in diagnosis of PCOS. In certain embodiments, the increase relative to a reference is at least 2.0, 3.0 or 4.0 fold, inclusive, and including all digits there between, and to the first decimal place. The reference value may also be a positive control value obtained from patients already diagnosed with the disease or disorder. In this case, if the measured value is the same or higher than the positive reference value, then it can be concluded

that the subject has the disease. If the measured value is lower, for example at least a statistically significant 1.5 fold decrease, then it can be concluded that the subject does not have the disease. In some embodiments, both positive and negative reference values are used in diagnosis. If it is concluded that the subject has the disease, then one can continue with a step or method of treatment as described herein.

[00069] Examples of biological samples include, but are not limited to; urine, blood, plasma, serum, and saliva. In some embodiments, the biological sample is a biopsy or surgical specimen of the ovary, endometrium, adipose tissue, or skeletal muscle. For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available and known in the art (e.g. various fluorimetric or colorimetric tags). In another embodiment of the invention, the anti-DENND1A.V2 antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the DENND1A.V2 antibody. The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays.

[00070] If DENNDIA Variant 2 mRNA is detected, this may be accomplished by a variety of techniques known in the art. Suitable techniques for determining the presence or absence or quantitating DENND1A Variant 2 mRNA include but are not limited to; hybridization of probes or primers directed to DENND1A Variant 2 mRNA, or by using various chip technologies, polynucleotide or oligonucleotide arrays, and combinations thereof. Thus, in various embodiments, probes to the DENND1A Variant 2 mRNA or a DNA equivalent of it can be arranged and/or fixed on a solid support.

[00071] DENND1A Variant 2 mRNA may be tested directly or may be amplified enzymatically *in vitro* by, for example, use of the polymerase chain reaction (PCR), Real-Time (RT) PCR, including quantitative real-time (qRT-PCR) PCR analysis, or any other in vitro amplification methods. For amplification reactions, primers can be designed which hybridize to and form a complex with DENND1A Variant 2 mRNA, and used to obtain nucleic acid amplification products (i.e., amplicons). Those skilled in the art will recognize how to design suitable primers and perform amplification and/or hybridization reactions in order to carry out various embodiments of the method of the invention. In general, the primers should be long enough to be useful in amplification reactions, and generally primers which are at least 12 bases in

length are considered suitable for such purposes; but primers as short as 8 bases can be used depending on reaction conditions. The primers/probes used for detecting DENND1A Variant 2 RNA can comprise modifications, such as being conjugated to one or more detectable labels; such as fluorophores in the form of a reporter dye and/or a quenching moiety for use in reactions such as real time (RT)-PCR, which allow quantitation of DNA amplified from RNA, wherein the quantitation can be performed over time concurrent with the amplification. In one embodiment, the amplification reaction comprises at least one polynucleotide probe specific for DENND1A Variant 2 mRNA, wherein the probe includes one terminal nucleotide modified to include a fluorescent tag, and the other terminal nucleotide modified to comprise a moiety that quenches fluorescence from the fluorescent tag. For instance, for use in qRT-PCR, such a probe can be designed so that it binds with specificity to a portion of DENNDA1 Variant 2 or its complement, that is between and does not overlap sequences to which two RT-PCR primers hybridize. Using this design, signal from the fluorescent tag will be quenched until the probe is degraded via exonuclease activity of the polymerase during amplification, at which point the fluorescent nucleotide will be separated from the quenching moiety and its signal will be detectable.

[00072] If desired, the determination of DENNDIA Variant 2 mRNA and/or protein can be compared to a reference value. The reference to which the Variant 2 mRNA and/or protein levels from the individual can be compared can be any suitable reference, examples of which include but are not limited to samples obtained from individuals who do not have the particular condition for which a diagnosis is sought, such as PCOS. Such references can include matched controls (i.e., matched for age, sex, or other demographics), a standardized curve(s), and/or experimentally designed controls such as known input RNA or protein used to normalize experimental data for qualitative or quantitative determination of the DENND1A Variant 2 from the sample for mass, molarity, concentration and the like. The reference level may also be depicted graphically as an area on a graph. In certain embodiments, determining Variant 2 mRNA and/or protein in a sample in an amount above a reference is a diagnosis of PCOS, or aids in a physician's diagnosis of PCOS. In certain embodiments, the reference is normal theca cells, which are compared to PCOS theca cells. In another embodiment, the reference is a sample that contains exosomes from an individual who does not have PCOS.

[00073] Before exemplary embodiments of the present invention are described in greater detail, it is to be understood that this invention is not limited to particular embodiments

described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[00074] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[00075] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, representative illustrative methods and materials are now described.

[00076] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[00077] It is noted that, as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

[00078] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and

features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[00079] The invention will be further illustrated by the following examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE 1. Overexpression of DENND1A.V2 produces a PCOS theca phenotype.

MATERIALS AND METHODS

[00080] *Normal and PCOS Theca Cells.* Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, following informed consent under a protocol approved by the Institutional Review Board of The Pennsylvania State University College of Medicine. Individual follicles were dissected away from ovarian stroma, dissected, and dispersed with 0.05% collagenase I, 0.05% collagenase IA, and 0.01% deoxyribonuclease, in medium containing 10% fetal bovine serum (FBS), as previously described [31]. The isolated follicles were size-selected for diameters ranging from 3-5 mm so that theca cells derived from follicles of similar size from normal and PCOS subjects could be compared. Theca cells were cultured on fibronectin coated dishes utilizing previously described growth medium (1:1 mixture of Dulbecco's Eagles Medium (DME) and Hams F-12 medium containing 5% FBS, 5% horse serum (HS), 2% UltroSer G™, 20 nM insulin, 20 nM selenium, 1 μM vitamin E and antibiotics). Sera and growth factors were obtained from the following sources: FBS and DME/F12 (Irvine Scientific®, Irvine, CA): horse serum (Life Technologies, Grand Island, NY); UltroSer G™ (Reactifs IBF, Villeneuve-la-Garenne, France): other compounds were purchased from Sigma® (St. Louis, MO). The cells were grown in reduced oxygen tension (5% O_2, 90% N_2, and 5% CO_2) and given supplemental antioxidants (vitamin E and selenium) to prevent oxidative damage.

[00081] The theca cell cultures utilized in these studies have been described and functionally characterized previously [15, 17, 32]. Experiments comparing PCOS and normal theca were performed utilizing 4th-passage (31-38 population doublings) theca cells isolated from size-matched follicles obtained from age-matched subjects. The use of fourth passage cells, propagated from frozen stocks of second passage cells in the media described above, allowed

multiple experiments to be performed from the same patient population. For all studies, theca cell cultures obtained from at least 5 independent normal and 5 independent PCOS patients were examined. The passage conditions and split ratios for all normal and PCOS cells were identical.

[00082] The PCOS and normal ovarian tissue came from age-matched women, 38-40 years old. The diagnosis of PCOS was made according to NIH consensus guidelines [10], which include hyperandrogenemia, oligoovulation, polycystic ovaries, and the exclusion of 21-hydroxylase deficiency, Cushing's syndrome, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than six menses per year and elevated serum total testosterone or bioavailable testosterone levels, as previously described [15, 33]. Each of the PCOS ovaries contained multiple subcortical follicles of less than 10 mm in diameter. The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21-35 days, and no clinical signs of hyperandrogenism. Neither PCOS nor normal subjects were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and/or pelvic pain.

[00083] *Western Blot Analysis.* Fourth passage normal and PCOS theca cells were grown until subconfluent and transferred into serum free medium with and without forskolin for 24 hours. Following treatment theca cells were harvested in ice cold modified RIPA buffer (30mM Tris, 150mM NaCl, 50 mM Na F, 0.5mM EDTA, 0.5% deoxycholic acid, 1.0% Nonident P-40, 0.1% SDS) containing 1 mM sodium orthovanadate, 0.1mM phenymethylsufonyl fluoride, 1 mM dithiothreitol, 0.2 mM benzamidine, 1μM microcystin, 1μg/ml leupeptin, and 1μg/ml pepstatin A. Protein concentration was determined using a BCA protein assay (Pierce, Rockford, Illinois, USA). Whole cell lysates (35 μg/ln) were separated on a 10% SDS-PAGE, transferred to PVDF membrane, and Western analysis was performed as previously described [32][34].

[00084] *Immunohistochemical Localization of DENND1A.V2.* Immunohistochemical analysis for DENND1A was performed on 4-5 μm thick sections of formalin-fixed, paraffin-embedded tissue on a Ventana Discovery XT stainer (Ventana Medical Systems, Tucson, AZ), using the Sigma DENND1A antibody found to be specific to DENND1A.V2. The staining protocol briefly consisted of incubation in the primary antibody for 30 minutes at 1:300 dilution followed by detection and visualization with Ventana OmniMap DAB anti-Rabbit detection kit (Ventana Medical Systems, Tucson, AZ). FFPE samples of adrenal cortex were used as a

positive tissue control [34]. A color image of the DENND1A.V2 immunohistochemistry has been published by McAllister et. al [34].

[00085] *Quantitative DENND1A.V1, DENND1A.V2, CYP17A1, and CYP11A1 Single Step Ultra-Fast qRT-PCR.* Quantitation of DENND1A.V1 and V.2, CYP17A1, and CYP11A1 mRNA abundance was determined using the Single Step Brilliant^{TM} III Ultra Fast qRT-PCR Reagents (Agilent) using 50-100 ng total RNA/tube, and 200nM final concentration of each forward and reverse primers, and 100nM probe. The specific primer and probe sequences used are provided in detail below. The "ZEN" moiety is shown as in the probe at an illustrative location but can be positioned elsewhere in the probe, as desired. The gene specific one step PCR was carried out in duplicate for each mRNA sample and for a series of dilutions in an Mx3000p Thermocycler system (Stratagene) according to manufacturer's instructions for this instrument. An arbitrary value of theca RNA template was assigned to each serial dilution (ie, 1000, 300, 100, 30, 10, 3, 1, 0.3, 0.1ng) and plotted against the Ct value (y-axis = Ct; x-axis = value, log scale) to generate a standard curve. Each unknown was assigned an arbitrary value based on the slope and y-intercept of the standard curve. The same process was carried out for TATA-box binding protein (TBP) in order to use TBP values for normalization of each reaction. The mean target value for each unknown was divided by the mean TBP value for each unknown to generate a normalized value for the target for each sample [34].

[00086] *Primer and Probe Sets*

The DENND1A Variant 2 specific mRNA Primer and Probe set[25] was specific to the 3'UTR, *Forward Primer (5' GGGCTGACTTCGGAGTGTGT 3')* (SEQ ID NO:34), *Reverse Primer (5' GGG CTG ACT TCG GAG TGT GT3')* (SEQ ID NO:35), *Probe (5'/56-FAM/CCAAAGAGC/ZEN/CGG TGT CTG ATA ATC CCA / 3iA3AbkFQ/-3')* (SEQ ID NO:36).

Second Confirmatory **DENND1A Variant 2 specific mRNA Primer and Probe** set was specific to the 33aa 3'CDS *Forward Primer (5' TCCACATGTTGTTAAGA GACCAAAG 3')* (SEQ ID NO:37), *Reverse Primer (5' CCGCAAAATGGGTAATGCTT 3')* (SEQ ID NO:38), *Probe (5'/56-FAM/AGCCCTGAG/ZEN/CAAAACACCATTGCAA/ 3iA3AbkFQ/-3')* (SEQ ID NO:39).

The DENND1A Variant 1 specific mRNA Primer and Probe set *Forward Primer (5'GGATTCATTTCCATAAACCAAGTTAAAG3')* (SEQ ID NO:40), *Reverse Primer*

(5'CACAATTTCCTGCGTGTCTCA3') (SEQ ID NO:41), *Probe (5'/56-FAM/ATGGCCCGA/ZENCCATTT AAGAAAACAACCA/#IA3BkFQ/-3)* (SEQ ID NO:42)

The *CYP17* mRNA Primer and Probe set. *Forward primer* (5'-GGCCTCAAA TGGCAAC TCTAGA-3') (SEQ ID NO:43), *Reverse Primer* (5'-CTTCTGATCGCCATCCTTGAA-3') (SEQ ID NO:44), *Probe(5' 6-FAM-TCGCGTCCAACAACCGTAAGGGTATC-3' BHQ-1)* (SEQ ID NO:45)

The CYP11A1 mRNA Primer and Probe set. *Forward primer* (5'*GAGGGAGACGGGCACACA*-3') (SEQ ID NO:46), *Reverse Primer* (5'- *TGACATAAA CCGACTCCACGTT* -3') (SEQ ID NO:47), *Probe(5' 6-FAM-TCCACCTTCACCATGTCCAGAATTTCCA-3' BHQ-1)* (SEQ ID NO:48)

The TATA-box binding protein (TBP) mRNA Primer and Probe set. TBP was also determined for each cDNA sample for normalization. *Forward Primer (5'-CACGGC ACTGATTTTCAGTTC-3')* (SEQ ID NO:49), *Reverse Primer (5'-TCTTGCTGCCAGTCTGGACT-3')* (SEQ ID NO:50), *Probe (5' JOE-TGTGCACAGGAGCCAAGAGTGAAGA-3' BHQ-1)* (SEQ ID NO:51).

[00087] *Quantitation of Steroid Biosynthesis.* ELISAs for dehyroepiandrosterone (DHEA), 17-hydroxyprogesterone (17OHP4), testosterone (T), and progesterone (P4) were performed without organic solvent extraction using kits from DRG International, Inc. (Springfield, NJ) as described by the manufacturer's protocol, and normalized by cell count.

[00088] *Exosomal RNA Extraction and Purification.* Mid-day urine samples were obtained from normal women and women with PCOS, using the same clinical criteria described above for normal and PCOS theca cells, following informed consent under an IRB protocol approved by the Institutional Review Board of The Pennsylvania State University College of Medicine. The urine samples are collected and placed at 4°C until processed, then aliquoted into 15 mL tubes and are centrifuged in a swing bucket in a Sorvall Super T21 Table Top Centrifuge at 300g at 4°C for 10 minutes to remove particulate matter. The supernatant is then serially centrifuged at 2000g at 4°C for 10 minutes to clear cellular debris, and the supernatant is transferred to a 17x100mm culture tube and centrifuged at 12,000g at 4°C for 30 minutes using a floor Beckman Coulter Avanti® J-E Centrifuge. This final cleared supernatant is frozen at -80°C, and is extracted using a modified protocol of the "Urine Exosome RNA Isolation Kit" from Norgen® (Thorold, CAN). The

resulting RNA is quantified using a Nano-drop. DENND1A.V2 mRNA was then quantitated by qRT-PCR, and normalized using 5S mRNA abundance [34]

[00089] *Replication-deficient Adenovirus Infections.* For these studies, DENND1A.V2 adenovirus (hDENND1A.V2-pADenoG) was obtained from Applied Biological Materials, Inc. (Vancouver, BC) that was constructed by cloning DENND1A.V2 from pCMV6-XL4 plasmid encoding the DENND1A.V2 into pADenoG, from Origene (Rockville, MD)[34]. Control empty NULL non-expressing adenovirus (pAdenoG Null) also obtained from Applied Biological Materials, Inc. (Vancouver, BC) [35-37]. These recombinant adenoviruses were propagated and expanded in HEK293T monolayer cells, purified using a Virabind™ Adenovirus Miniprep Kit, (Cell Biolabs, Inc, San Diego, CA), and titered by QuickTiter Adenovirus Titer Elisa Kit, (Cell Biolabs, Inc., San Diego, CA). Both the DENND1A.V2 or Control empty NULL non-expressing adenovirus (pAdenoG Null) were used to infect normal theca cells as previously described [34] [38]. Adenoviral infections involved growing fourth passage theca cells to 75% confluence, rinsing the cells with phosphate buffered saline, and layering of the adenovirus on the cells in 50% of the normal treatment volume of serum free medium for 60 min. Subsequently, the cells were cultured in serum-free media with and without treatment as indicated.

[00090] *Transient transfection of normal and PCOS theca cells.* Human theca cells isolated from normal cycling women and women with PCOS were transfected as previously described [17, 22, 39]. One hour prior to transfection, the cells were transferred into DME high-glucose medium containing 20 mmol/L HEPES and 2% heat-inactivated calf serum (Atlanta Biologicals, Inc., Atlanta, GA) and moved to a 3% CO_2, 95% ambient air, 37°C incubator. The calcium phosphate precipitate contained 2 μg/dish of luciferase plasmid, and 0.1 μg/dish of an expression vector for β-galactosidase, pSVβ-gal (Promega, Madison, WI) for each 30 mm well. Following transfection, the cells were rinsed with 15% glycerol/HBSS followed by PBS, and treated in transfection media containing vehicle or 10 μM forskolin for 48 h. Cells were harvested with trypsin, pelleted, and resuspended in reporter lysis buffer (Promega). Luciferase activity was determined with the Luciferase Assay System (Promega) on a Sirius Luminometer (Zylux Corp., Oak Ridge, TN). β-Galactosidase activity was measured by the chemiluminescent assay Galacto-Light Plus™ (Tropix, Bedford, MA) and utilized for normalization of transfection efficiency. Transfections were performed in triplicate in theca cells isolated from 4 or more independent normal control

patients, and 4 or more independent PCOS patients unless otherwise noted in the text of this Example.

[00091] *Statistical Analysis.* Data are presented and described in this Example as the mean ± SEM performed triplicate. The results from qRT-PCR, steroid, and transfection analysis were collected from individual patients and *ANOVA* was performed using Prism 5.0c (GraphPad Software, San Diego, CA). P values were determined by the Boniferri method for multiple comparisons when significant differences were indicated by ANOVA [34].

[00092] *DNA Extraction.* DNA was extracted from theca cell cultures isolated and propagated from independent normal cycling (n = 6) and PCOS women (n = 6). The frozen theca cell culture plates were retrieved from -80° C and 1 mL of lysis solution, containing 0.10 μL 1M Tris-HCl pH 8.0 (Invitrogen, Carlsbad, CA), 0.01 μL 0.5M EDTA pH 8.0 (Promega, Madison, WI), 0.02 μL 10% SDS (Fisher Biotech, Fair Lawn, NJ), 0.04 μL 5M NaCl (Promega, Madison, WI), 0.01 μL 10 mg/mL Proteinase K (Life Technologies™, Carlsbad, CA), and 0.82 mL of water, was added to each plate[34]. Plates were scraped to retrieve cells in solution. The lysis solution containing the cells was transferred to 1.5 mL microcentrifuge tubes, which were incubated in a tube rotator at 55° C overnight. Samples were centrifuged at 12,000 rpm for 5 min at room temperature. Approximately 400 μL of sample was transferred to 1.5 mL microcentrifuge tube containing 500 μL of isopropyl alcohol and inverted several times to precipitate DNA. DNA was washed twice with 70% EtOH and transferred to microcentrifuge tube containing 400 μL of 1X Tris-EDTA pH 7.4 (Fisher BiorReagents, Fair Lawn, NJ). Tubes were incubated in a tube rotator at 55° C for 2 h to dissolve DNA in solution[34].

[00093] *Whole Exome Sequencing.* The DNA samples were subjected to whole exome sequencing at 100 millions reads, at 100X coverage using the Agilent SureSelect 51M capture kit with Illumina HiSeq 2000 sequencing, in conjunction with BGI Americas (Cambridge, MA).

[00094] *Exome Sequence Analysis.* Sequence reads obtained were aligned to the reference genome (Human hg19) by BGI [34]. Variant calling and summary of data production was performed by BGI. Visualization of data and identification of called variants was done using the IGV (Integrative Genomic Viewer) software [40]. Only those variants with support numbers > 15 (count of the uniquely mapped base) for the minor allele were selected from the normal and PCOS theca cell samples for further analysis. SNPs in DENND1A were identified and their minor allele frequencies were calculated. Data is presented in Table 1[34].

[00095] Table 1 shows the DENND1A variants identified by Whole Exome Sequence Analysis of DNA extracted from independent normal cycling (n = 6) and PCOS women (n = 6): The table lists the variants along with rs identifiers for snps, previously listed and identified in dbSNPs, chromosome locations, position/ function of the variant in the gene, reference allele in human reference genome Hg19. The minor allele frequency of the snp in our sample set (6 Normal, 6 PCOS) were calculated and compared with the allele frequency in European American population if present[34].

Table 1: DENND1A variants identified by Whole Exome Sequence Analysis of DNA extracted from independent normal cycling and PCOS women.

Gene DENND1A	Location					Calculated minor allele freq		
SNP	Chr	Position	Function	Ref Allele	# Samples with SNP	Normal	PCOS	EA
rs3739837	9	126143662	utr-3	G	1 N, 1 PCOS	A (0.083)	A (0.083)	A (0.1109)
rs2491348	9	126144746	Synonymous -coding	T	6 N, 6 PCOS	C (1.0)	C (1.0)	T (0.0003)
rs2808409	9	126144758	Synonymous -coding	T	6 N, 6 PCOS	C(1.0)	C(1.0)	ABSENT
rs2808411	9	126146197	Intron	C	6 N, 6 PCOS	G (1.0)	G (1.0)	C (0.082)
rs10739631	9	126164562	Intron	T	6 N, 6 PCOS	C (1.0)	C (1.0)	ABSENT
rs7872778	9	126201672	Intron	G	1 N, 0 PCOS	A (0.083)	Absent	ABSENT
rs62581072	9	126201742	Intron	G	0 N, 1 PCOS	Absent	A (0.083)	ABSENT
rs10739633	9	126202551	Intron	A	5 N, 5 PCOS	C (0.75)	C (0.75)	ABSENT
rs3829851	9	126219706	Synonymous -coding	A	1 N, 0 PCOS	G (0.083)	Absent	G (0.055)
snp4115	9	126219742	Intron	C	0 N, 1 PCOS	Absent	T (0.083)	ABSENT
snp3842	9	126220063	Intron	G	0 N, 1 PCOS	Absent	T (0.083)	ABSENT
rs61736953	9	126220114	Synonymous -coding	C	1 N, 2 PCOS	T (0.083)	T (0.166)	T(0.0884)
rs12377595	9	126439100	Intron	T	2 N, 3 PCOS	G (0.166)	G (0.33)	G (0.2490)
rs9785285	9	126520068	Synonymous -coding	T	3 N, 2 PCOS	C (0.33)	C (0.166)	C (0.2123)
rs41274356	9	126531724	Intron	T	1 N, 0 PCOS	C (0.083)	Absent	C (0.0239)
rs1778890	9	126531755	Intron	T	3 N, 2 PCOS	C (0.33)	C (0.166)	C (0.2148)
snp3895	9	126554844	Intron	A	0 N, 1 PCOS	Absent	G (0.083)	ABSENT
rs670028	9	126641091	Intron	A	2 N, 0 PCOS	G (0.25)	Absent	ABSENT

[00096] *Exome Data Comparison.* Minor allele frequencies of the DENND1A variants identified by our WES analysis were compared to the allele frequencies of these variants in the population using the online database – Exome 6500 [34]. Comparisons were made with the European American population frequencies since the theca cell samples in this study were extracted from patients of Western European descent [34].

RESULTS

[00097] **DENND1A.V2 protein expression is increased in PCOS theca cells**. To examine whether the alternatively spliced forms of DENND1A are differentially expressed in normal and PCOS theca cells[34], Western Blot analysis was performed on whole cell extracts from theca cells isolated from normal and PCOS women, that were grown until subconfluent and transferred into serum free medium treated with and without 20 μM forskolin for 24 h. An intermediate N-terminal DENND1A antibody (Sigma) was utilized, and we expected bands at approximately 112 kD, corresponding, to DENND1A.V1, and a 62 kD band, corresponding to DENND1A.V2. As shown in Fig 1A, representative Western Blot analysis demonstrated increased 62 kD DENND1A.V2 in PCOS theca cells treated under both basal and forskolin-stimulated conditions compared to normal theca cells. However, the 112 kD DENND1A.V1 was not significantly increased in PCOS theca cells. In Fig 1B, cumulative analysis of whole cell lysates harvested from theca cells isolated from 5 independent normal and 5 independent PCOS subjects, demonstrated that DENND1A.V2 protein was increased (*, $P < 0.01$) in PCOS theca cells as compared to normal theca cells under both control and forskolin-stimulated conditions. Forskolin treatment did not appear to affect DENND1A.V2 protein accumulation in normal or PCOS theca cells. In contrast, cumulative analysis of DENND1A.V1 in normal and PCOS theca cells, showed that DENND1A.V1 protein level was increased (*, $P < 0.01$) by forskolin treatment in normal cells (Fig 1C). In addition, in forskolin-stimulated PCOS theca cells, DENND1A.V1 was significantly reduced compared to normal cells. The ratio of DENND1A.V2/V1 protein was increased (**, $P < 0.01$) in PCOS theca cells under control and forskolin stimulated conditions (Fig 1D)[34]. Similar results were obtained in Western analyses performed using Abcam antibody specific for the N-terminal and Sigma antibody for intra-peptide sequence of DENND1A. DENND1A Western data were quantitated and normalized by total mTOR which is

not significantly different in normal and PCOS theca cells, nor regulated by forskolin treatment. Actin and GAPDH cannot be utilized to normalize Westerns as they are regulated by forskolin in theca cells, and are differentially expressed in normal and PCOS cells.

[00098] Western analyses were also performed to evaluate the efficacy of a rabbit polyclonal antibody, generated against the 21 amino acid peptide (QKSITHFAAKFPTRGWTSSSH)(SEQ NO:6) that is specific to DENND1A.V2. Western Blot analysis was performed using whole cell extracts from theca cells isolated and propagated from normal and PCOS women, that were treated with and without 20 μm forskolin for 24 h in serum free media. As shown in Fig 1E, representative Western Blot analysis demonstrated an increase in 62 kD DENND1A.V2 in PCOS theca cells treated under both basal and forskolin-stimulated conditions. In these experiments, protein loading was normalized by total mTOR. These data are in agreement with parallel Western blot analyses presented in Fig 1A with the N-terminal DENND1A antibody. As shown in Fig 1F, representative Western Blot analysis demonstrated increased 62 kD DENND1A.V2 in PCOS theca cells treated under both basal and forskolin stimulated conditions. In Fig 1F, cumulative analysis of whole cell lysates harvested from theca cells isolated from 4 independent normal and 4 independent PCOS patients, demonstrated that DENND1A.V2 protein is significantly increased in PCOS theca cells as compared to normal theca cells under both control and forskolin-stimulated conditions (*, $P < 0.01$). Forskolin treatment did not appear to affect DENND1A.V2 protein accumulation in normal or PCOS theca cells.

[00099] **DENND1A.V2 Immunohistochemical Staining is Increased in the Ovarian Theca Compartment.** To further examine the localization of DENND1A.V2 in the ovary, the paraffin embedded blocks of ovarian tissue obtained from the normal cycling and PCOS patient populations, and an antibody specific to DENND1A.V2 was utilized[34]. As shown in Fig 2A immuno-staining was prominent in the theca compartment of the ovary (4X), and increased in PCOS theca as compared to theca cells in normal ovaries (10X). In Fig 2B, DENND1A.V2 staining in PCOS theca and granulosa cells is shown (40X). Staining is primarily in the PCOS theca nuclei, cytoplasm, and cell membranes as shown (100X, Oil mag) in Fig 2B [34].

[000100] **DENND1A.V2 mRNA abundance is increased in PCOS theca cells compared to normal theca cells, and correlates with increased androgen production by PCOS theca cells.** The abundance of DENND1A.V2 mRNA was examined in theca cells isolated from 6

normal and 6 PCOS patients treated with and without 20 μM forskolin (Fig 3A), quantitated by QRT-PCR analysis, and normalized using TATA box binding protein (TBP) mRNA. DENND1A.V2 mRNA was significantly increased under basal and forskolin-stimulated conditions in PCOS theca cells, as compared to normal cells. There was no significant increase in DENND1A.V2 mRNA accumulation in response to forskolin stimulation. To examine whether DENND1A.V2 mRNA was associated with increased androgen biosynthesis, we examined DHEA accumulation in the matched 6 normal and 6 PCOS theca cell preparations under the same conditions. As shown in Fig 3B, normal theca cells with lower DHEA synthesis have reduced DENND1A.V2 mRNA, whereas the increase in basal and forskolin stimulated DHEA production in PCOS theca cells is associated with increased DENND1A.V2 mRNA abundance [34].

[000101] **Urine exosomal DENND1A.V2 mRNA is increased in PCOS women.** Exosomes are small nucleic acid rich vesicles that are shed into blood and urine, and provide a stable source of RNA for use in personalized medicine and clinical diagnostics. Thus, to examine whether increased DENND1A.V2 in PCOS theca cells reflects corresponding increases in systemic DENND1A.V2 mRNA accumulation in PCOS women, exosomal mRNA isolated from mid-day urine samples from multiple normal cycling and well-characterized PCOS women was examined. Urine exosomal mRNA was isolated and prepared, as described in Materials and Methods, from mid-day urine samples from normal cycling and PCOS women. As shown in Fig 4, and in agreement with the qRT-PCR in PCOS theca cells, DENND1A.V2 mRNA is significantly increased in urine exosomes isolated from PCOS, as compared to normal cycling women [34].

[000102] **Forced Expression of DENND1A.V2 Increases Normal Theca Cell Steroidogenesis.** An adenovirus expressing human DENND1A.V2 was utilized to test the hypothesis that increased expression of DENND1A.V2 converts normal theca cells to a PCOS phenotype of increased androgen and progesterone production. In these experiments, normal theca cells were infected with 0.3, 1.0, 3.0, and 10 pfu/cell of either empty (Null-pAdenoG) or DENND1A.V2 expressing (hDENND1A.V2-pADenoG) adenovirus, treated with or without 20 μM forskolin in serum free medium. Following 72h of treatment, DHEA in the media was quantitated. Infection with all doses of DENND1A.V2 adenovirus significantly increased forskolin-stimulated DHEA production compared with control adenovirus (Fig 5A). In

subsequent experiments, the effects of infection of theca cells from several individual normal women with 3.0 pfu of control adenovirus or adenovirus expressing DENND1A.V2 on DHEA (Fig 5B), 17OHP4 (Fig 5C), T (Fig 5D), and P4 (Fig 5E) was investigated. DENND1A.V2 adenovirus infection significantly increased basal 17OHP4, T, and P4 accumulation compared with control adenovirus. In addition, DENND1A.V2 adenovirus infection significantly increased forskolin-stimulated DHEA, 17OHP4, and P4 compared with control adenovirus. For these experiments Western analysis confirmed that DENND1A.V2 protein was increased following forced expression (data not shown) [34]. Thus, forced expression of DENND1A.V2 in normal theca cells, converted the cells to a PCOS phenotype of increased androgen and progestin biosynthesis [15, 16, 34].

[000103] Forced expression of DENND1A.V2 Increases *CYP17A1 and CYP11A1* gene expression in Normal Theca Cells. To examine the effects of DENND1A.V2 expression on CYP17 and CYP11A1 mRNA accumulation, cultures of 4th passage normal theca cells were infected with 3 pfu DENND1A.V2 adenovirus, or Null adenovirus, and treated with or without 20 μM forskolin for 16h. Following treatment, RNA was harvested, and CYP17 and CYP11A1 mRNA abundance was quantitated and normalized by TBP abundance. Both CYP17A1 mRNA (Fig 6A) and CYP11A1 mRNA (Fig 6B) accumulation were significantly increased following 3 pfu/cell DENND1A.V2 infection under forskolin-stimulated conditions, as compared to infection with control adenovirus. These finding were also confirmed using transfection of normal theca cells with a DENND1A.V2 expression plasmid (DENND1A.V2/pCMV-XL4) obtained from Origene (Rockville, MD)[34], as well as following DENND1A.V2 lentiviral particle infection (data not shown).

[000104] To examine the effects of DENND1A.V2 on *CYP17A1* transcription, normal theca cells were transfected with pGl3 luciferase reporter plasmid containing -770/+44 of the 5'-flanking regions of the human *CYP17A1* gene *(-770 CYP17A1*/LUC) using the calcium phosphate method [17, 22, 34]. Following transfection the cells were infected with the DENND1A.V2 adenovirus or control adenovirus for 1h, and treated with serum-free medium with and without 20 μM forskolin, an activator of adenylate cyclase. 24h thereafter luciferase activity was determined. Transfections were performed in triplicate, normalized for transfection efficiency using β-galactosidase. Fig 6C shows that 3 pfu/cell DENND1A.V2 adenovirus

infection increases both basal and forskolin-stimulated *-770CYP17A1/LUC* promoter activity as compared to control adenovirus [34].

[000105] A *CYP11A1* pGl3 reporter construct containing the -160/-90bp element of the proximal *CYP11A1* promoter (*-160/-90 CYP11A1*/LUC) which confers increased *CYP11A1* expression in PCOS theca cells was used to examine the effects of DENND1A.V2 on *CYP11A1* transcription in theca cells. Normal theca cells were transfected with the *-160/-90 CYP11A1*/LUC plasmid with a DENND1A.V2/pCMV-XL4 or empty pCMV-XL4. 48h following transfection the cells were harvested and luciferase was measured. As shown in Fig 6D, DENND1A.V2 increases forskolin-stimulated *-160/-90 CYP11A1/LUC* promoter activity as compared to empty plasmid. Collectively, these data suggest that the increased theca cell steroidogenesis resulting from augmented DENND1A.V2 expression is due at least in part to transcriptional activation of the *CYP17A1* and *CYP11A1* genes [34].

[000106] **Knock-down of DENND1A.V2 mRNA in PCOS Theca Cells Reduces *CYP17A1* and *CYP11A1* Expression, Androgen and Progesterone Production.** To determine the effect of knock-down of endogenous DENND1A.V2 mRNA on CYP17A1 and CYP11A1 mRNA levels, silencing DENND1A.V2 shRNA plasmids were transfected into PCOS theca cells, and basal and forskolin stimulated CYP17A1 mRNA was assessed using qRT-PCR. In these experiments 4th passage PCOS theca cells were transfected with pRSV-Scrambled plasmid or plasmids specific to DENND1A.V2 (pSV-shRNA1 or pRSV-shRNA2). 6h following transfection, the cells were treated with and without 20 μM forskolin, and 24h thereafter, total RNA was harvested, and CYP17A1, CYP11A1 and TBP mRNA abundance was measured. As shown in Figure 7A, DENND1A.V2 shRNA1 and shRNA2 retrovirus plasmid significantly inhibited both basal and forskolin stimulated CYP17A1 mRNA accumulation in PCOS theca cells. Both of the DENND1A.V2 shRNA plasmids also significantly inhibited forskolin-stimulated CYP11A1 mRNA (Fig 7B) in PCOS theca cells [34]. For these experiments Western and qRT-PCR analyses confirmed that DENND1A.V2 protein was significantly decreased following DENND1A shRNA transfection [34].

[000107] Parallel studies were performed to evaluate whether silencing shRNAs specific to DENND1A.V2 would inhibit *CYP17A1* promoter function (i.e., transcription) in PCOS theca cells. PCOS theca cells were transfected with a *CYP17A1* luciferase reporter plasmid (-235 *CYP17A1*/LUC) containing -235/+44 of the *CYP17A1* promoter fused to the luciferase gene in

pGL3. Scrambled pRV expression vector or plasmid encoding the silencing DENND1A.V2 pRV-shRNA1 or pRV-shRNA2 was also added to the transfection mixture. 6h following transfection, the cells were rinsed with PBS, and treated in serum-free medium with and without 20 μM forskolin. 24h thereafter luciferase activity was determined. The results from these experiments showed that transfection of DENND1A.V2 shRNA1 inhibited basal and cAMP-dependent *CYP17A1* reporter activity in PCOS theca cells, compared to scrambled shRNA. An increase in both basal and forskolin-stimulated -235 *CYP17A1*/LUC promoter regulation in PCOS theca cells was observed. As shown in Fig 7C, co-transfection of -235*CYP17A1*/LUC with a silencing DENND1A.V2 shRNA1 or shRNA2 resulted in a significant reduction of forskolin-dependent *CYP17A1* reporter activity in PCOS theca cells, compared to Scrambled shRNA [34].

[000108] Silencing of DENND1A.V2 Expression by shRNA Lentivirus Particles Inhibits PCOS Theca Cell Steroidogenesis. To evaluate the effect of knockdown of DENND1A.V2 on steroid biosynthesis, custom Thermo/ Dharmacon GIPZ DENND1A.V2 shRNA particles were utilized. PCOS theca cells were infected with 300,000 particles/well of silencing shRNA DENND1A.V2 lentivirus or a control non-silencing lentivirus in serum free medium[34]. Six hours thereafter the lentivirus mixture was removed the cells were transferred into serum free medium in the presence or absence of forskolin for 72 h. Infection with silencing shRNA DENND1A.V2 lentivirus significantly inhibited forskolin-stimulated 17OHP4 (Fig 7D), DHEA biosynthesis (Fig 7E), and P4 (Fig 7F). Although there was a trend towards inhibition in basal steroidogenesis following DENND1A.V2 lentivirus infection, the design of lentiviral infection experiments precluded the accurate assessment of basal steroid concentrations.

[000109] Rabbit polyclonal Anti-DENND1A.V2 Specific IgG Reduces DHEA Secretion and CYP17A1 mRNA in PCOS Theca Cells. DENND1A is known to be associated with the cell membrane and clathrin coated pits, potentially making DENND1A epitopes available to antibodies added to the cell exterior. We generated a rabbit polyclonal antibody against a 21 amino acid peptide (QKSITHFAAKFPTRGWTSSSH) (SEQ ID NO:6) that is specific to DENND1A.V2 (Fig 1C-D). This DENND1A.V2 specific IgG was added to the culture medium of normal and PCOS theca cells to determine whether it could be used to block or neutralize DENND1A.V2 function, and thus alter steroid biosynthesis in normal and PCOS theca cells. In these experiments PCOS theca cells were treated with increasing concentrations of a

DENND1A.V2 isoform specific IgG or non-specific IgG, in the presence or absence of 20 μM forskolin. As shown in Fig 8A, DENND1A.V2 IgG significantly inhibits forskolin-stimulated DHEA biosynthesis, with an approximate ID_{50} of 0.25 μg/mL. In contrast, non-specific IgG had no effect on DHEA biosynthesis[34].

[000110] Subsequent experiments were performed to examine the effects of 0.5 μg/mL of rabbit polyclonal DENND1A.V2 specific IgG or 0.5 μg/mL non-specific IgG on CYP17A1 mRNA (Fig 8B) and CYP11A1 mRNA (Fig 8C) accumulation following 16 h treatment in the presence and absence of 20 μM forskolin in normal and PCOS theca cells from various individual patients [34]. The data in Fig 8B demonstrate that 0.5 μg/mL DENND1A.V2 specific IgG significantly inhibits CYP17A1 mRNA accumulation in PCOS theca cells, under control and forskolin-stimulated conditions following 16h of treatment. In contrast, in normal theca cells, DENND1A.V2 IgG had no effect on basal or forskolin-stimulated CYP17A1 mRNA (Fig 8B). As shown in Fig 8C, DENND1A.V2 specific IgG also significantly inhibited forskolin-stimulated CYP11A1 mRNA accumulation in PCOS theca cells, while having no effect on CYP11A1 mRNA accumulation in normal theca cells.

[000111] In parallel experiments, normal and PCOS theca cells from individual patients were treated with 0.5 μg/mL of affinity purified rabbit polycolonal DENND1A.V2 specific IgG or 0.5 μg/mL or non-specific IgG and basal and forskolin stimulated absence DHEA (Fig 8D), 17OHP4 (Fig 8E), and P4 (Fig 8F) biosynthesis on a per cell basis was measured following 72 h treatment. The results of these experiments showed that rabbit polyclonal DENND1A.V2 specific IgG significantly inhibited forskolin-stimulated DHEA (Fig 8D) and 17OHP4 (Fig 8E), in PCOS theca cells by 50% as compared to control IgG, without affecting normal theca cells.

DISCUSSION

[000112] It will be apparent from the foregoing that we have carried out the first studies to examine the expression of DENND1A in well characterized theca cells from normal cycling and PCOS women. The discovery that increased expression of a splice variant of DENND1A mRNA, DENND1A.V2, is characteristic of PCOS theca cells provided a basis for pursuing studies on the functional consequences of this molecule in terms of theca cell function [34].

[000113] Forced expression of DENND1A.V2 in normal theca cells increases *CYP17A1* and *CYP11A1* gene expression and converts the cells to a PCOS phenotype of augmented androgen and progestin biosynthesis. In contrast, knock-down of DENND1A.V2 with silencing

shRNA plasmids or lentivirus in PCOS theca cells reverts the cells to a normal phenotype of reduced *CYP17A1* and *CYP11A1* gene expression and androgen and progestin biosynthesis. These observations suggest that DENND1A is involved in a signaling cascade that augments transcription of steroidogenic genes that subsequently results in increased androgen production.

[000114] DENND1A.V2 is a truncated connecdenn 1, which has a clathrin-binding domain and is thought to facilitate endocytosis. Among the loci associated with PCOS in Han Chinese [41-43], several reside in or near genes that potentially define a network, including the FSHR, LHCGR, and INSR, which encode receptors that reside on the plasma membrane, and which are internalized by clathrin coated pits, where DENND1A protein is located [25, 42, 44]. The DENN domains of DENND1A function as Rab-specific guanine nucleotide exchange factors [26]. Ras related protein 5B (RAB5B), another PCOS GWAS candidate, is a Rab-GTPase, also thought to be involved in endocytosis and receptor recycling and could, therefore, be a molecule interacting with the DENN domain [45, 46]. RAB5B signaling has also been reported to involve PI3K, PKB, and MAPK/ERK components [44-47]. Without being bound by theory, it is possible that the truncated form of DENND1A.V2 affects insulin or LH-receptor turnover and sensitivity in theca and/or granulosa cells, further affecting ovarian function and steroid biosynthesis in PCOS women. DENND1A has also been shown to be associated with lipids, particularly phosphoinsitol-3-phosphate, and other endocytosis/endosome proteins [26], and could potentially be involved in insulin and LH-receptor signaling. Alternatively, DENND1A.V2 may have a more direct role in controlling gene expression. DENND1A.V2 may be translocated into the nucleus, a process that may be facilitated by RAB5B, another GWAS discovered PCOS candidate, where it may be involved in transcriptional activation of *CYP17A1* and *CYP11A1* gene expression [34].

[000115] Using specific rabbit polyclonal, and human and mouse monoclonals anti-DENND1A.V2 IgG, it has been shown in the Examples herein that DENND1A.V2 epitopes are available to extracellular antibodies. The observation that augmented CYP17A1 and CYP11A mRNA and androgen biosynthesis in PCOS theca cells can be reduced using DENND1A.V2 specific IgG indicates that biologic agents such as humanized monoclonal antibodies against DENND1A.V2 are useful therapeutic agents for the hyperandrogenemia associated with PCOS, and possibly other phenotypes related to insulin action.

[000116] These findings demonstrate that a splice variant (DENND1A.V2) derived from the *DENND1A* gene, has a functional role in controlling theca cell steroidogenesis [34]. Overexpression of DENND1A.V2 is sufficient to convert normal theca cells into a PCOS biochemical phenotype characterized by increased *CYP17A1* and *CYP11A1* gene expression and augmented androgen and progestin production. Suppression of DENND1A.V2 function pushes PCOS theca cells towards a normal phenotype in terms of steroidogenic enzyme gene expression and steroid production. The fact that DENND1A.V2 mRNA is elevated in urine of women with PCOS and that a DENND1A.V2-specific IgG can transform the biochemical characteristics of PCOS theca cells, reducing steroidogenesis, indicates the usefulness of DENND1A.V2-specific antibodies for non-invasive detection of PCOS and also for biological therapy of PCOS [34]

EXAMPLE 2. Humanized monoclonal antibodies specific for DENND1A.V2 isolated from a phage display library are useful for the treatment of PCOS.

MATERIALS AND METHODS

[000117] **Phage Display Screening.** To obtain a functional humanized mAB specific to DENND1A.V2, a newly made Phage Display Scl-2 Library (Creative Biolabs, New York) was screened [48-54]. The antibody and antigen binding fragments were intended to target the unique 33 amino acid C-terminus of the DENND1A.V2 protein. The 33 amino acid DENND1A.V2 sequence that is unique between DENND1A Variants 1 and 2 is: NTIATPATLHILQKSITHFAAKFPTRGWTSS SH (SEQ ID NO:5). For panning, the unique 21 amino acid sequence segment: QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) was utilized. This is the sequence that was previously used to obtain a functional rabbit polyclonal antibody to DENND1A.V2 (see Example 1).

Materials used for Phage Display

A newly prepared phage display Scl-2 Library
E. coli TG1 host strain
M13KO7 helper phage:
Primers:
L1 :5'-TggAATTgTgAgCggATAACAATT-3' (SEQ ID NO:54)
S6 :5'-gTAAATgAATTTTCTgTATgAgg-3' (SEQ ID NO:55)
Target : CH22, DENND1A.V2
Rabbit anti-M13 pAb-HRP

[000118] Initially, two DENND1A variant 2 peptide-protein conjugates [KLH- and BSA-conjugate] to QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) were utilized to perform phage display panning to select specific binders to the peptide itself. After coating the peptide-BSA conjugate directly, affinity panning and QC phage ELISA were conducted. The results showed that no specific enriching effect was observed.

[000119] A biotinylated version of the DENND1A.V2 specific peptide (QKSITHFAAKFPTRGWTSSSH; SEQ ID NO:6) was synthesized, and new panning was performed with a freshly made phage library as described in Table 2 by immobilizing the bio-peptide with strepavidin pre-coated plates. A strong enriching effect was observed after three rounds of screening. 40 clones were picked up either from the trypsin-digestion eluate or the competitive eluate, and subjected to phage amplification and phage ELISA (Tables 3-4).

Table 2. Human Phage Panning process for CH22 DENND1A.V2 amino acid sequence target QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) (i.e., Biotin-CH22)

Round	*Conditions*	*Input*	*Output*	*Enriching factor*
1st	Target protein: 100 ug/ml Biotin-CH22 Washing: 0.1 % Tween20 PBST, 10 times Elution: Trypsin digestion Pre counter select：2%M-PBS	1.10×10^{11}	2.48×10^{5}	4.44×10^{5}
2nd-P	Target protein: 60 ug/ml Biotin-CH22 Washing: 0.2 % Tween20 PBST, 10 times Elution: Trypsin digestion Pre counter select：2%M-PBS	1.60×10^{11}	8.32×10^{5}	1.92×10^{5}
2nd-N	Target protein: no coating Washing: 0.2% Tween20 PBST, 10 times Elution: Trypsin digestion Pre counter select：2%M-PBS	4.00×10^{10}	3.00×10^{3}	1.33×10^{7}
3rd-P1	Target protein:40 ug/ml Biotin-CH22 Washing: 0.2 % Tween20 PBST, 10 times Elution: Trypsin digestion Pre counter select：2%M-PBS	1.60×10^{11}	7.98×10^{6}	2.01×10^{4}

3rd-N	Target protein: no coating Washing: 0.2% Tweenß20 PBST, 10 times Elution: Trypsin digestion Pre counter select：2%M-PBS	4.00×10^{10}	4.78×10^{4}	8.37×10^{5}
3rd-P2	Target protein:40 ug/ml Biotin-CH22 Washing: 0.2 % Tween20 PBST, 10 times Elution: Competitive digestion Pre counter select：2%M-PBS	1.60×10^{11}	1.01×10^{6}	1.58×10^{5}
3rd-N	Target protein: no coating Washing: 0.2% Tween20 PBST, 10 times Elution: Competitive digestion Pre counter select：2%M-PBS	4.00×10^{10}	5.50×10^{3}	7.27×10^{6}

Enriching factor=input/output

Table 3. QC monoclonal phage ELISA-1. Reactivity of the human M13K07 monoclonal phage clones to the biotinylated DENND1A.V2 amino acid sequence QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) (i.e., Biotin-CH22)

M13KO7 phages: 10(3) cfu/ml

Clones	Coating: Biotin-CH22	No coating
1	0.140	0.101
2	0.228	0.109
3	0.128	0.099
4	0.123	0.099
5	0.127	0.125
6	0.115	0.128
7	0.134	0.100
8	0.115	0.105
9	0.159	0.096
10	0.273	0.108
11	0.143	0.096
12	0.142	0.094
13	0.147	0.088
14	0.223	0.095
15	0.422	0.196
16	**1.669**	**0.089**
17	0.130	0.133
18	0.221	0.100
19	0.124	0.121
20	0.127	0.111
21	0.170	0.102
22	0.137	0.102
23	0.167	0.102
24	0.230	0.094
25	0.135	0.110
26	0.135	0.085
27	0.140	0.085

28	0.179	0.104
29	0.165	0.089
30	0.125	0.098
31	0.140	0.109
32	0.164	0.074
33	0.121	0.096
34	0.196	0.102
35	0.248	0.142
36	0.179	0.108
37	0.118	0.085
38	0.126	0.111
39	0.151	0.104
40	0.146	0.089
1-A	0.311	0.091
2-A	0.731	0.103
3-A-1	0.877	0.077
3-A-2	0.951	0.070
M13KO 7	0.477	0.074
1%M-PBS	0.081	0.066

1-A : Amplified phages of the 1st eluate
2-A: Amplified phages of the 2nd eluate
3-A: Amplified phages of the 3rd eluate

Table 4. QC monoclonal phage ELISA-2. Reactivity of the human M13K07 monoclonal phage clones to the biotinylated DENND1A.V2 amino acid sequence QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) (i.e., Biotin-CH22).

Clones	Coating: Biotin-CH22	No coating
41	0.184	0.096
42	0.193	0.108
43	0.178	0.116
44	0.179	0.096
45	0.218	0.102
46	0.200	0.099
47	0.238	0.100
48	0.176	0.101
49	0.158	0.103
50	0.221	0.114
51	0.165	0.097
52	0.181	0.094
53	0.220	0.141
54	0.141	0.098
55	0.161	0.097
56	0.169	0.101
57	0.229	0.111

58	0.208	0.144
59	0.345	0.157
60	0.326	0.116
61	0.167	0.115
62	0.213	0.159
63	0.190	0.130
64	0.162	0.106
65	0.278	0.081
66	0.180	0.120
67	0.227	0.101
68	0.369	0.122
69	0.270	0.098
70	0.223	0.129
71	0.199	0.105
72	0.164	0.115
73	0.222	0.107
74	0.214	0.109
75	0.154	0.102
76	0.289	0.100
77	0.176	0.094
78	0.184	0.113
79	**1.535**	**0.112**
80	0.279	0.102
M13KO7	0.533	0.114
1%M-PBS	0.115	0.072

M13KO7: 10(10) cfu/ml

[000120] Using the biotinylated DENND1A.V2 peptide QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) as the target, two strong binding scFv's (clones 16 and 79) were identified (Tables 3-4). Sequence analysis of scFv phage clones 16 and 79 showed that the nucleotide and peptide sequences of these clones were identical (Fig 9A-B). The VL and VH of this scFV were cloned into plasmids containing the human light (pIgG1-L) and heavy chains (pIgG1-H) of IgG1 (Fig 10A-D). Following transfection, these constructs were expressed in HEK293 cells, and the resulting human recombinant DENND1A.V2 IgG1 was expressed and purified (Fig 11A-C). Briefly, a plasmid vector was transfected into 293E cells. The suspension culture was collected 96 hours after transfection. The product was purified by HiTrap rProteinA FF and filtered using a 0.2μm filter. The resulting human recombinant DENND1A.V2 specific IgG (V2 hmAB) (Fig 11B) was then utilized to perform functional studies using ovarian androgen producing human theca cells isolated from normal cycling women and women with PCOS (Fig 12A-B and Fig 13A).

RESULTS

[000121] **Phage display results**. As shown in Table 2, a strong enriching effect was found after three rounds of screening. Accordingly, 40 clones were picked up either from the trypsin-digestion eluate or the competitive eluate for further QC ELISA assay. The first 40 clones from the trypsin-digestion eluate were subjected to QC phage ELISA. As shown in Table 3, one strong positive clone, clone 16, were identified together with a few weak positive clones. The second 40 clones from the competitive eluate were also subjected to QC phage ELISA. As shown in Table 4, a second strong positive clone, clone 79, was identified with a few weak positive clones. DNA sequencing was performed on the two strong positive clones and several weak positive binding clones. 7 unique scFvs were identified. Two strong positive clones (clones 16 and 79) shared the same scFv sequence (see Fig 9).

[000122] **Soluble scFv expression and soluble scFv ELISA.** The strong positive scFv gene (identical clones 16, 79) was cloned into a soluble scFv expression vector pCDisplay-2 for further soluble scFv expression and soluble scFv ELISA. As shown in Table 5, when the *E. coli* lysate containing soluble scFv-AP fusion proteins was coated directly, expression was successfully confirmed. As shown in Table 6, when biotinylated DENND1A.V2 peptide was immobilized via a streptavidin pre-coated plate, the positive binding of scFv-AP was confirmed as well.

Table 5. ELISA analysis of soluble scFv fusion proteins from phage clones 16 and 79 using E coli lysate directly.

Clones	30°C	37°C
16-①	1.934	0.682
16-②	1.681	0.882
79-①	1.821	1.161
79-②	1.975	0.943
TG1	0.098	
1%M-TBS	0.130	

Table 6. QC soluble scFv ELISA using immobilize DENND1A.V2 biotinylated peptide via strep-avidin pre-coating plate.

Clones	Temp.	Coating: Biotin-CH22	No coating
16-①	30°C	2.132	0.191
	37°C	2.102	0.206
16-②	30°C	2.118	0.179
	37°C	2.172	0.160
79-①	30°C	2.144	0.222
	37°C	2.144	0.196
79-②	30°C	2.142	0.212
	37°C	2.100	0.211
TG1	---	0.150	0.139
1%M-TBS	---	0.121	0.130

[000123] The VL and VH of the scFv identified from phage display, were cloned into plasmids containing the human light (pIgG1-L) and heavy chains (pIgG1-H) of IgG1, and sequenced. As shown in Figs 10A-D the VH and VL of the heavy and light chains of these clones were identical to those of the scFv (Fig 9A-9B). The CDRs of both the VH and VL are underlined and bracketed.

[000124] **Recombinant human DENND1A.V2 specific IgG1 functionally inhibits DHEA biosynthesis in PCOS theca cells.** Dose response experiments were performed to examine whether the recombinant human IgG1 obtained from phage display screening with the biotinylated DENND1A.V2 21 amino acid peptide (QKSITHFAAKFPTRGWTSSSH)(SEQ ID NO:6) could neutralize the effects of augmented DENND1A.V2 expression and reduce steroid biosynthesis in PCOS theca cells (Fig 12). In these experiments PCOS theca cells were treated with increasing concentrations (.01-6 μg/mL) of a human recombinant monoclonal IgG1 specific for DENND1A.V2 (V2 hmAB) or a non-specific IgG1, treated in the absence (C) and presence of 20μM forskolin (F) for 72h. As shown in Figure 12A, there was a dose dependent decrease in DHEA biosynthesis. V2 hmAB significantly decreased (*, $P<0.01$) forskolin-stimulated DHEA biosynthesis with an approximate ID50 of 1.8 μg/mL, compared to control IgG1. Examination of the effects of 3.0 μg/ml and 6.0 μg/ml non-specific IgG1 or V2 hmAB on PCOS theca cell biosynthesis, showed that both basal and forskolin (*, $P<0.1$) stimulated DHEA biosynthesis were inhibited following treatment with V2 hmAB as compared to control IgG1

(Fig 12B). In parallel preliminary studies, human recombinant DENND1A.V2 IgG1 also inhibited basal and forskolin-stimulated CYP17 mRNA accumulation (data not shown).

[000125] Subsequent experiments were performed to examine the effects of a maximal dose of human recombinant DENND1A.V2 IgG1 (V2 hmAB) (9 μg/ml) and non-specific IgG1 (9 μg/ml) on androgen biosynthesis in normal and PCOS theca cells from various individual patients, treated in the absence (C) and presence of 20 μM forskolin, for 72 h. As shown in Figure 13A, a maximal dose of V2 hmAB IgG1 significantly (*, $P<0.01$) inhibited forskolin-stimulated DHEA biosynthesis in PCOS theca cells, while having minimal effect on DHEA accumulation in normal theca cells.

[000126] A comparison of the effects of rabbit DENND1A.V2 polyclonal (Fig 8) and human recombinant DENND1A.V2 IgG1 (Fig 12A-B and 13A) on DHEA biosynthesis demonstrated that both antibodies generated against the C-terminal DENND1A.V2 21 amino acid peptide have reduced basal and forskolin- stimulated androgen biosynthesis in PCOS theca cells, as compared to control IgG (Fig 13B)

EXAMPLE 3. Mouse monoclonal antibodies specific for DENND1A.V2 provides the CDR sequence information of a functional IgG1 for the construction of humanized monoclonals for the therapeutic treatment of PCOS.

MATERIALS AND METHODS

[000127] **Production of mouse monoclonal antibodies to DENND1A peptide.** Mouse monoclonal antibodies to the DENND1A peptide QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6) were prepared using standard procedures [55]. BALB/c mice were immunized using KLH-coupled peptide in RIBI adjuvant. Immunizations were delivered both subcutaneously and intra-peritoneally in volumes of 0.05 ml per site per mouse per immunization. Immunizations were given bi-weekly 3 times, the final booster immunization was given as KLH-peptide in saline. Three days after the final booster immunization, the mice were anesthetized using ketamine/xylazine and spleen and lymph nodes removed following exsanguination. Single cell suspensions of immune cells were prepared and fused with P3X63-Ag8.653 myeloma cells for the production of hybridomas. Supernatants from cultures of hybridomas were screened by ELISA for reactivity to the peptide, and to BSA-peptide and positive cultures isolated for expansion and cloning. Positive clones producing reactive antibodies in ELISA were adapted to

serum free conditions using Sigma serum-free culture media and then high titer antibodies were produced using BDBiosciences CELLine devices. Cultures containing antibody were then purified using Protein A/G columns (Pierce, Rockford, IL) and used in the various antibody detection and function assays.

[000128] **ELISA binding activity of a panel of mouse monoclonal antibodies.** ELISA was conducted to assess binding characteristics of selected monoclonal antibodies that appeared positive in the primary screening of the fusions. The basic method is to coat ELISA plate wells with various DENND1A.V2 antigen sources (KLH-peptide; BSA-peptide, free peptide, control peptide and BSA) at 1 ug/ml in Bicarbonate binding buffer (pH 9.6) then test a single dilution of hybridoma cell culture supernatant in duplicate. The optical density readings at 405nm were established from a plate reader, and mean $\pm$ SEM of OD readings plotted for each antigen and each hybridoma supernatant. Some of the reactive hybridomas were cloned by limiting dilution, isotype characterized, and retested in the ELISA. An example of the binding profiles of the mouse monoclonal antibodies by ELISA are shown in Fig 14. Several monoclonal antibodies were identified to have specific reactivity to DENND1A.V2 free peptide (SEQ ID NO:6) as well as KLH- and BSA labeled peptide, including P1B2, P1C5, P4F1, P5F9, and P2F5.

[000129] **RNA Extraction and Sequence Analysis of the Mouse DENND1A.V2 Monoclonal Antibodies.** ELISA-positive monoclonal hybridoma cells were pelleted by centrifugation and re-suspended in TRIzol Reagent (Life Technologies) for RNA isolation. Total RNA was extracted from the homogenate and the resulting RNA pellet was dissolved in 100 µl HyClone HyPure Molecular Biology Grade Water (Thermo Scientific). cDNA was synthesized using the RevertAid First Strand cDNA synthesis kit which included both the random hexamer primers and the recombinant M-MuLV RT used for the synthesis. The resulting cDNA was amplified by ChoiceTaq DNA Polymerase (Denville Scientific Inc.) and IgG1 heavy chain redundant and kappa light chain redundant primers [55][56]. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Using the same primers as was used for PCR amplification, PCR products were directly sequenced by Operon. Sequences were analyzed and translated using DNAMAN software and sequenced using the same PCR primers.

[000130] **Functional Evaluation of the mouse monoclonal antibodies on androgen biosynthesis.** The hybridoma cells from the P1B2 and P1C5 mouse monoclonal clones were further subcloned, and the culture media from these cultures were utilized to treat PCOS theca

cells, to evaluate their effects on ovarian androgen biosynthesis in PCOS theca cells as described in Example 1, Material and Methods.

[000131] **Sequence Analysis of the mouse DENND1A.V2 monoclonal antibodies.** RNA was prepared from the PIB2 and P1C5 hybridoma clones. A partial nucleotide and amino sequence of the heavy and light chains of mAb P1B2, and heavy chains of P1C5 are shown in Figs 16A-D.

RESULTS

[000132] **Mouse DENND1A.V2 mAB ELISA.** An ELISA scan of mouse monoclonal antibody supernatants for selected hybridomas identified several monoclonal antibodies that had specific reactivity to DENND1A.V2 free peptide (SEQ ID NO:6) as well as KLH- and BSA labeled peptide, including P1B2, P1C5, P4F1, P5F9, and P2F5 (Fig 14)

[000133] **Inhibitory effects of mouse monoclonal V2 IgG1 on androgen biosynthesis.** Experiments were performed to investigate whether the mouse P1B2 (mV2.A) and P1C5 (mV2.B). DENND1A.V2 monoclonal antibodies that were generated against KLH- conjugated DENND1A.V2 specific 21 amino acid peptide (QKSITHFAAKFPTRGWTSSSH) (SEQ ID NO:6), could similarly neutralize DENND1A.V2 in PCOS theca cells and suppress androgen biosynthesis. In these experiments, PCOS theca cells were treated with 40 μL of tissue culture supernatant of either mV2.A and mV2.B mouse IgG1, or negative control mouse IgG1 in the presence and absence of 20 μM forskolin for 72h. The data in Figure 14 demonstrate that both DENND1A.V2 specific mV2.A and mV2.B mouse IgG1 inhibit forskolin-stimulated DHEA biosynthesis in PCOS theca cells (*, $P < 0.05$) as compared to negative control mouse IgG1. The data in Figures 12-13 and 15 demonstrates that both human and mouse DENND1A/V2 specific antibodies suppress DHEA biosynthesis in PCOS theca cells. This is in agreement with results obtained using rabbit DENND1A.V2 specific polyclonal antibody (Fig 8). These data further support the notion that monoclonal antibodies specific to DENND1A.V2 could be used as a therapeutic modality for PCOS.

[000134] **Sequences of the DENND1A.V2 specific mouse monoclonal antibodies P1B2 and P1C5.** Both the DNA and protein sequences of the mouse monoclonal DENND1A.V2 specific IgG1, P1B2 heavy chain (Fig 16A and C; SEQ ID NOs: 60 and 62), P1B2 light chain (Fig 16B and D; SEQ ID NOs:61 and 63), and the heavy chain of P1C5 (Fig 16E-F; SEQ ID NOs: 64 and 65) nucleotide and amino acid sequences are provided. The CDRs are bracketed.

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While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims. Accordingly, the present invention should not be limited to the embodiments as described above, but should further include all modifications and equivalents thereof within the spirit and scope of the description provided herein.

CLAIMS

We claim:

1. A pharmaceutical composition comprising an antibody or an antigen binding fragment thereof, wherein said antibody or antigen binding fragment thereof specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein.

2. The pharmaceutical composition of claim 1, wherein said antigen binding fragment is selected from the group consisting of Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fd fragments, Fv fragments, scFv fragments, and combinations thereof.

3. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof specifically recognizes at least one epitope present in the amino acid sequence: NTIATPATLHILQKSITHFAAKFPTRGWTSSSH (SEQ ID NO:5).

4. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof specifically recognizes at least one epitope present in the amino acid sequence: QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6).

5. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof specifically recognizes at least one epitope comprising at least 4 contiguous amino acids in the sequence NTIATPATLHILQKSITHFAAKFPTRGWTSSSH (SEQ ID NO:5) and/or the sequence QKSITHFAAKFPTRGWTSSSH (SEQ ID NO:6).

6. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof specifically recognizes at least one epitope present in at least one of the following amino acid sequences: NTIATPA; TIATPAT; IATPATL; ATPATLH; TPATLHI; PATLHIL; ATLHILQ; TLHILQK; LHILQKS; HILQKSI; ILQKSIT; LQKSITH; QKSITHF; KSITHFA; SITHFAA; ITHFAAK; THFAAKF; HFAAKFP; FAAKFPT; AAKFPTR; AKFPTRG; KFPTRGW; FPTRGWT; PTRGWTS; TRGWTSS; RGWTSSS; and GWTSSSH (SEQ ID NO:7 to SEQ ID NO:33).

7. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof contains an amino acid sequence comprising residues 1-116 and 133-240 of SEQ ID NO:53.

8. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof contains an amino acid sequence comprising the complementary determining regions (CDRs) of SEQ ID NOs: 62 and 63.

9. The pharmaceutical composition of claim 1, wherein said antibody or antigen binding fragment thereof contains an amino acid sequence comprising the CDRs of SEQ ID NO: 65.

10. An isolated nucleic acid encoding an antibody or an antigen binding fragment thereof, wherein said antibody or antigen binding fragment thereof specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein.

11. The nucleic acid of claim 10, wherein said nucleic acid comprises a sequence represented by SEQ ID NO:56 and/or SEQ ID NO:57.

12. The nucleic acid of claim 10, wherein said nucleic acid comprises a sequence represented by SEQ ID NO:60 and/or SEQ ID NO:61.

13. The nucleic acid of claim 10, wherein said nucleic acid comprises a sequence represented by SEQ ID NO:64.

14. A method for the treatment of a disorder that is positively correlated with expression of DENND1A.V2 mRNA and/or protein comprising

administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical composition comprising an antibody or an antigen binding fragment thereof, wherein said antibody or antigen binding fragment thereof specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein.

15. The method of claim 14, wherein said disorder is polycystic ovary syndrome (PCOS).

16. The method of claim 15, wherein said disorder is type II diabetes associated with PCOS.

17. A method for altering signaling by cell surface receptors in cells having increased DENND1AV.2 expression as compared to control cells comprising

contacting said cells with an antibody or antigen binding fragment thereof, wherein said antibody or antigen binding fragment thereof specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein.

18. The method of claim 17, wherein said cells are selected from the group consisting of theca cells, adipose cells, skeletal muscle cells, endometrial cells, and granulosa cells.

19. The method of claim 18, wherein contacting said cells with said antibody or antigen binding fragment thereof decreases androgen and/or progesterone biosynthesis in said cells and/or decreases gene expression of *CYP17A1* and/or *CYP11A1* mRNA and/or protein in said cells.

20. The method of claim 19, wherein said decreasing of the androgen and/or progesterone biosynthesis in theca cells and/or said decreasing of the gene expression of *CYP17A1* and/or *CYP11A1* mRNA and/or protein in said cells reduces or prevents symptoms of excess androgen production and/or anovulation associated with hyperandrogenemia.

21. The method of claim 20, wherein said symptoms of excess androgen production comprise hirsutism.

22. The method of claim 18, wherein said antibody or antigen binding fragment thereof reduces or prevents other phenotypes associated with PCOS selected from the group consisting of abnormal insulin signaling, insulin resistance in adipose, skeletal muscle, and endometrial tissue, abnormal FSH signaling in granulosa cells, and combinations thereof.

23. An article of manufacture comprising a container and a composition contained therein, wherein said composition comprises an antibody which specifically recognizes DENND1A.V2 protein and does not specifically recognize DENND1A.V1 protein and further comprising an insert indicating that the composition can be used to treat a disorder that is positively correlated with expression of DENND1A.V2 mRNA and/or protein.

24. The article of manufacture of claim 23, wherein said disorder is PCOS.

25. A method for diagnosis of a disorder that is positively correlated with expression of DENND1A.V2 mRNA and/or protein in a subject comprising

contacting a biological sample from said subject with an antibody wherein said antibody contains an amino acid sequence comprising residues 1-116 and 133-240 of SEQ ID NO:53 or the CDRs of at least one of SEQ ID NOs: 62, 63, or 65; and

comparing the amount of DENND1A.V2 detected to a reference value.

26. The method of claim 25, wherein said disorder is PCOS.

27. The method of claim 25, wherein said biological sample is blood, serum, urine, plasma, or saliva and/or a biopsy or surgical specimen of the ovary, endometrium, adipose tissue, or skeletal muscle.

28. A method for the treatment of a disorder that is positively correlated with expression of DENND1A.V2 mRNA and/or protein comprising

administering to a subject in need thereof a therapeutically effective amount of a RNAi containing composition, wherein said RNAi composition decreases the expression of DENND1A.V2.

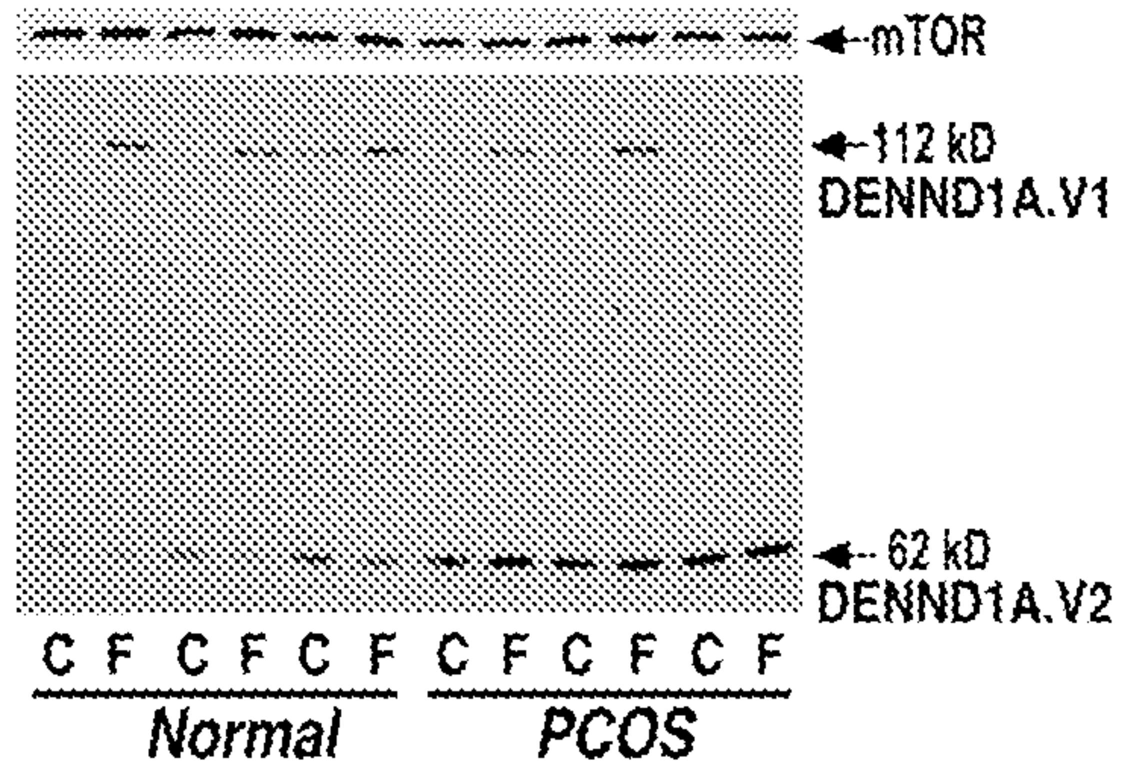


Figure 1A

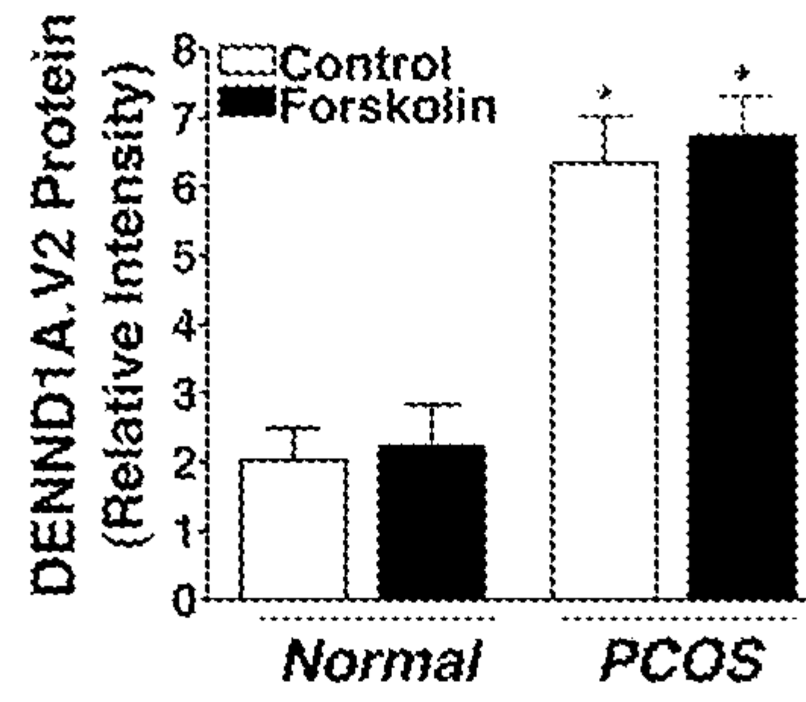


Figure 1B

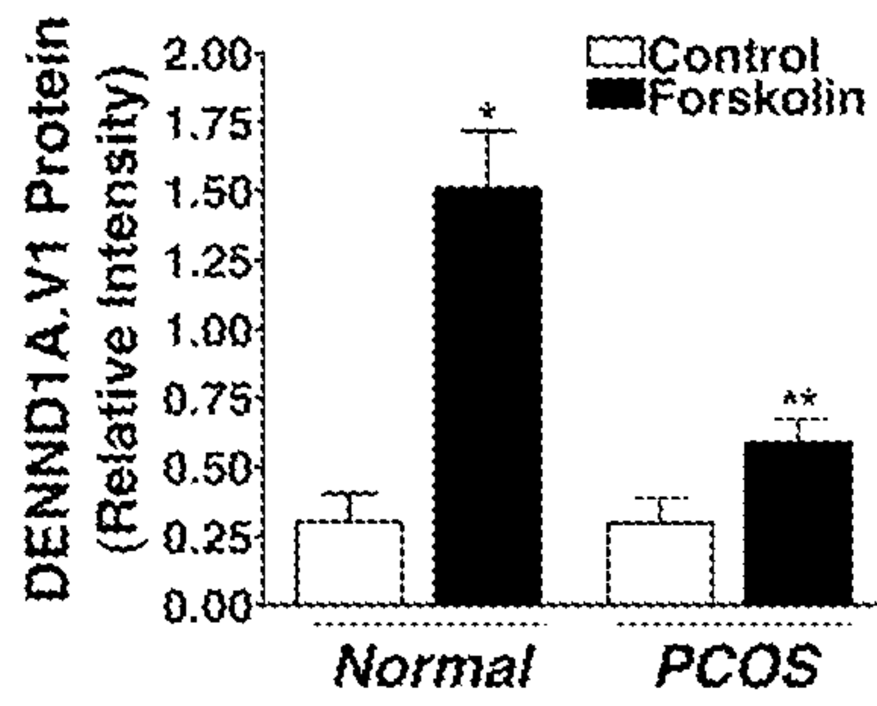


Figure 1C

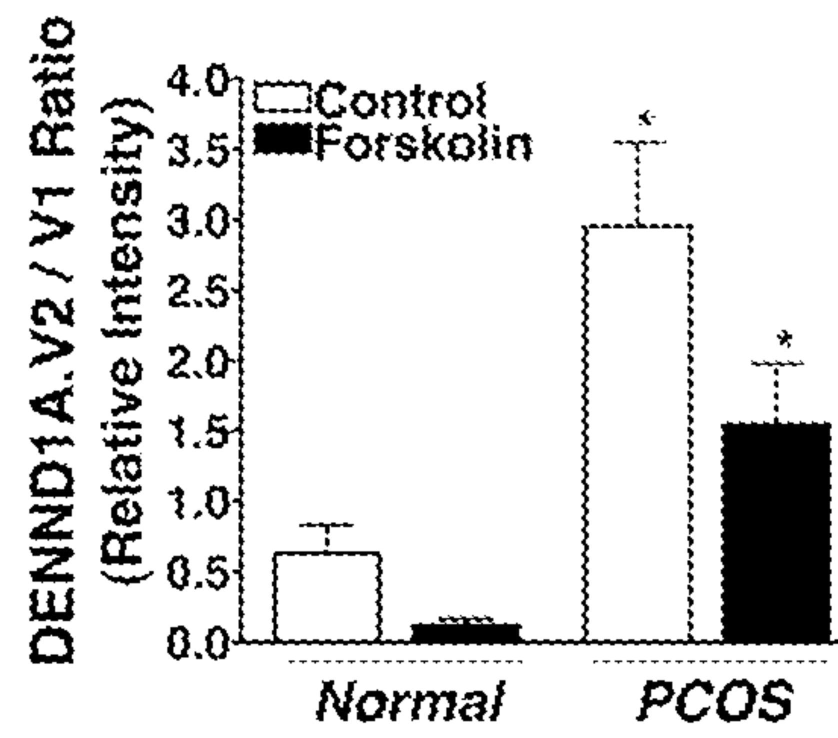


Figure 1D

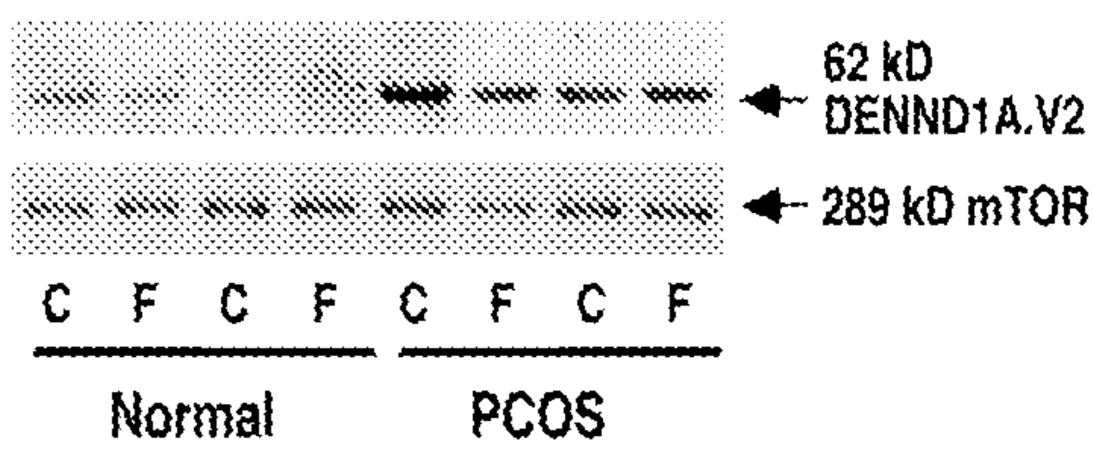


Figure 1E

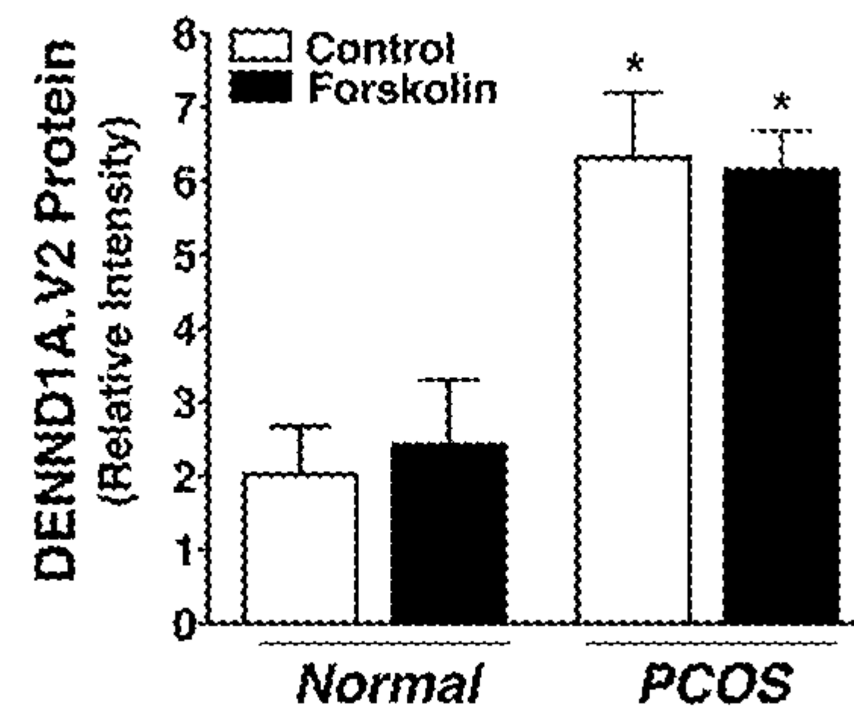


Figure 1F

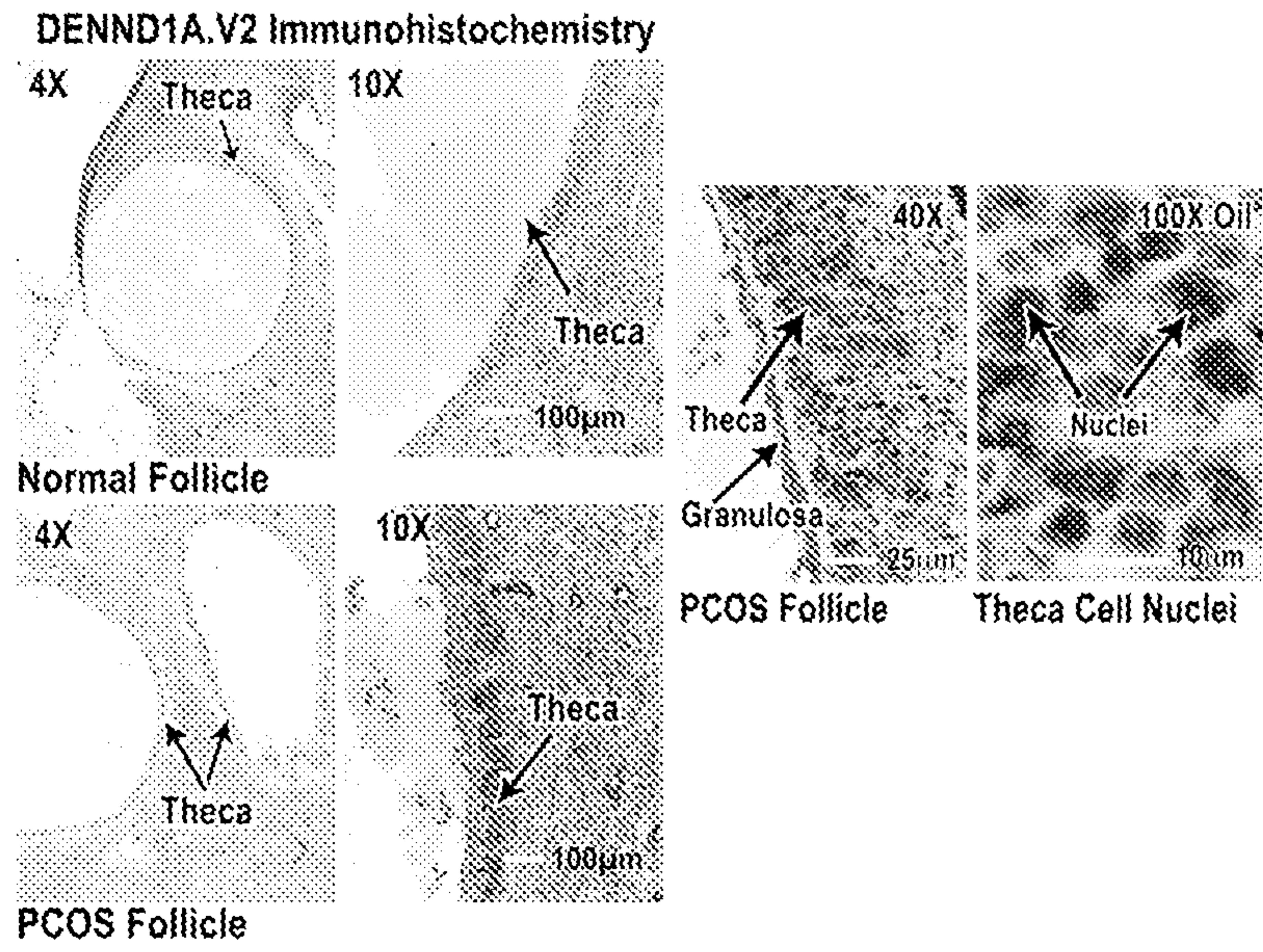


Figure 2A **Figure 2B**

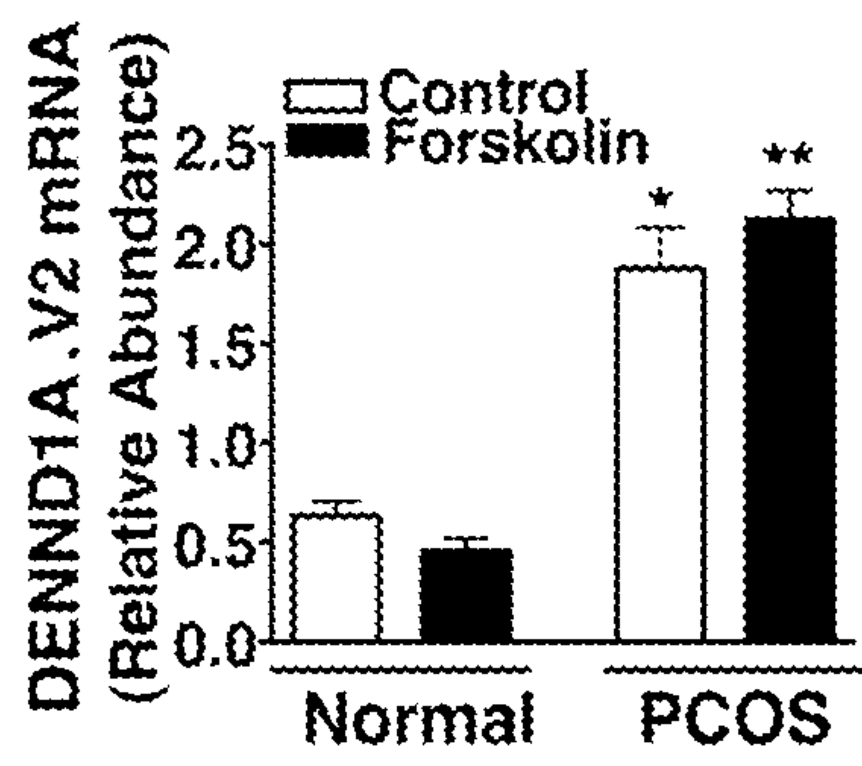


Figure 3A

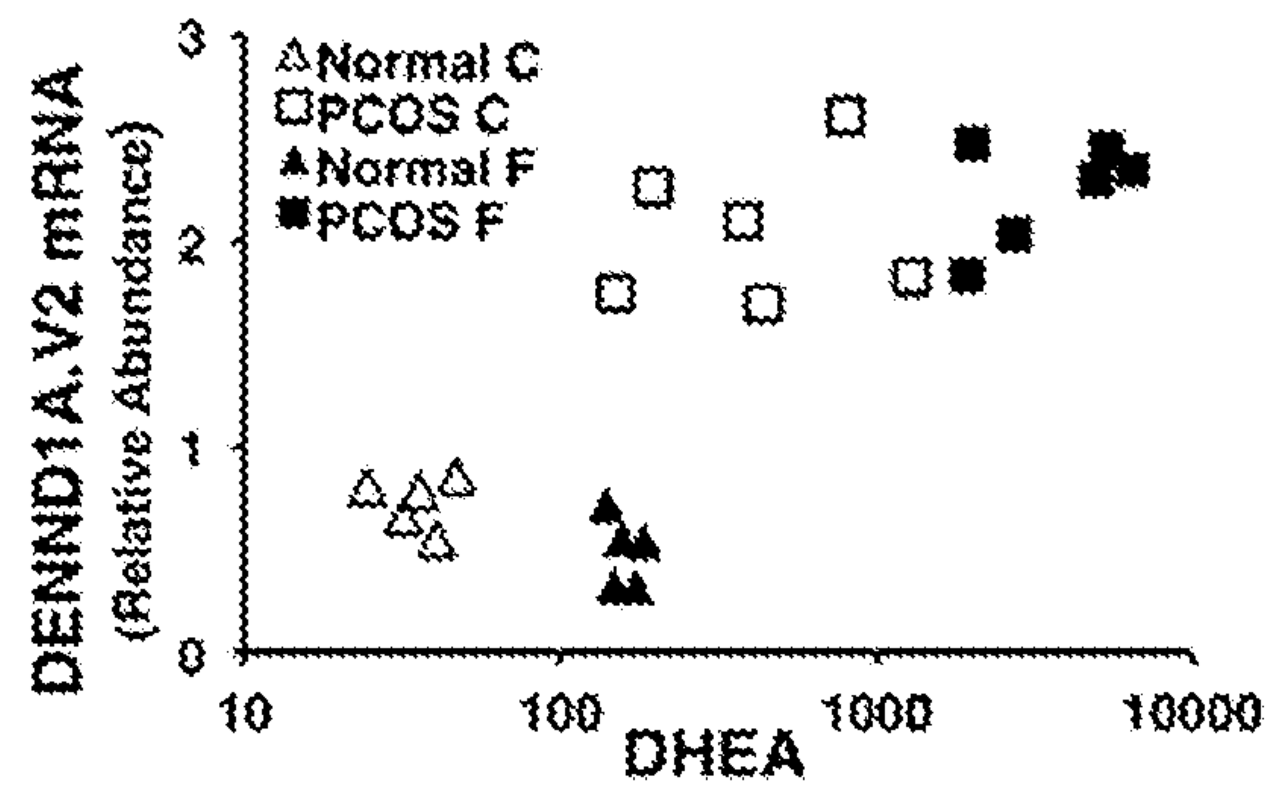


Figure 3B

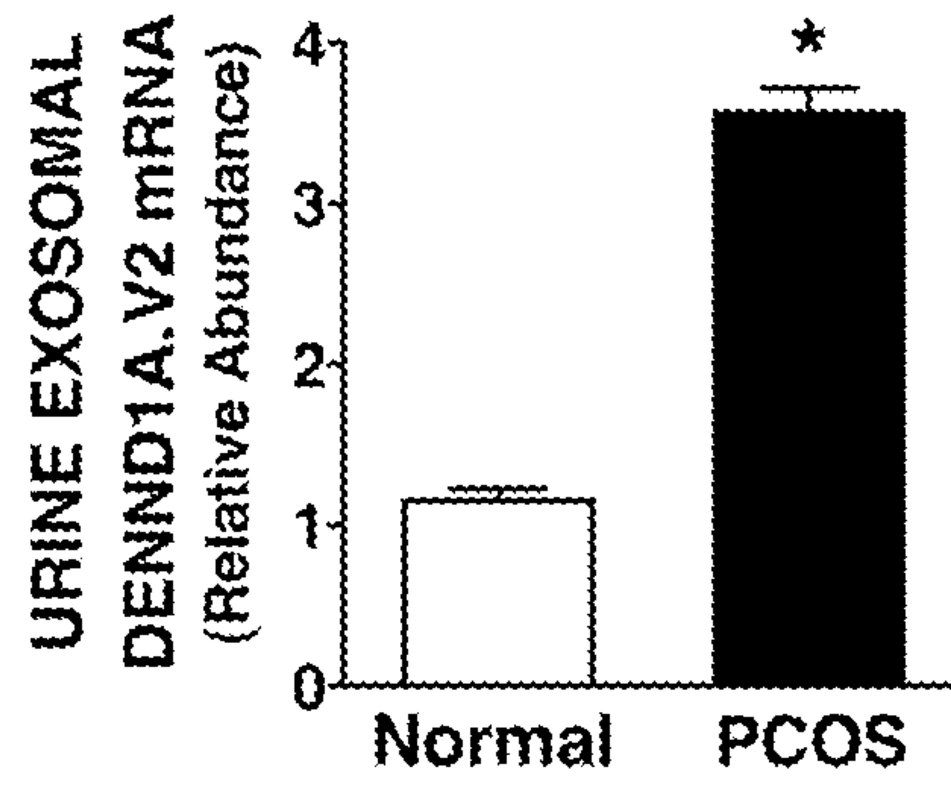


Figure 4

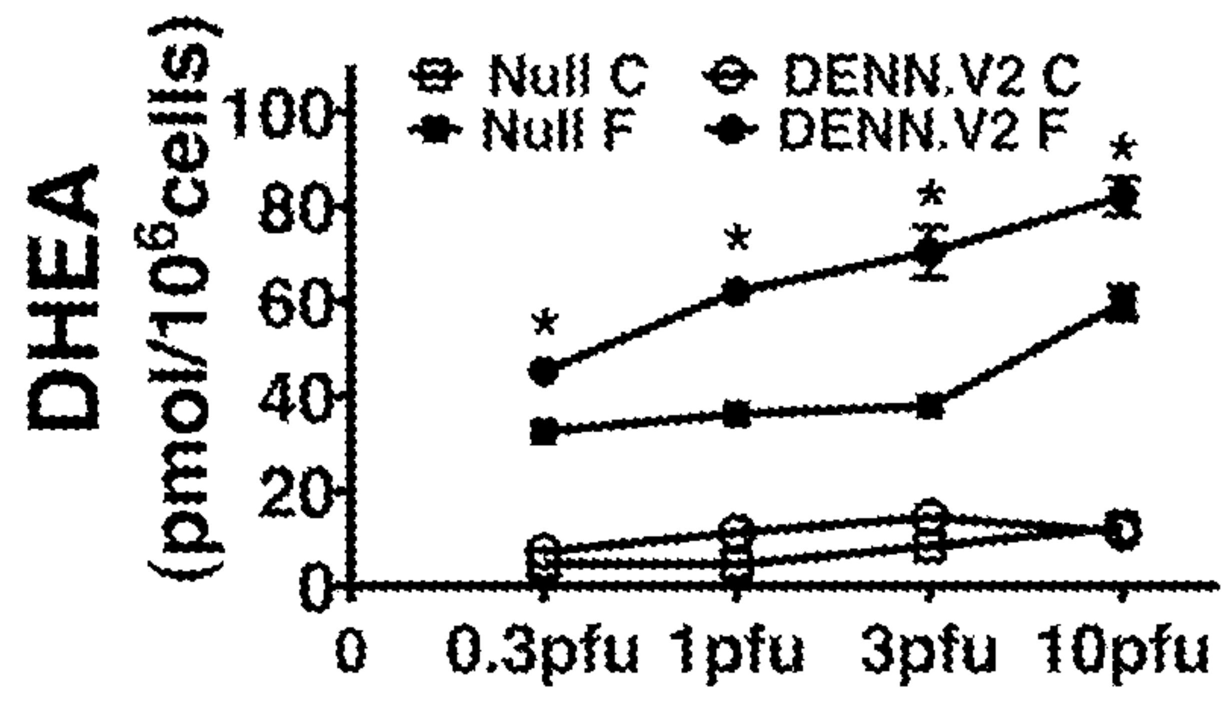


Figure 5A

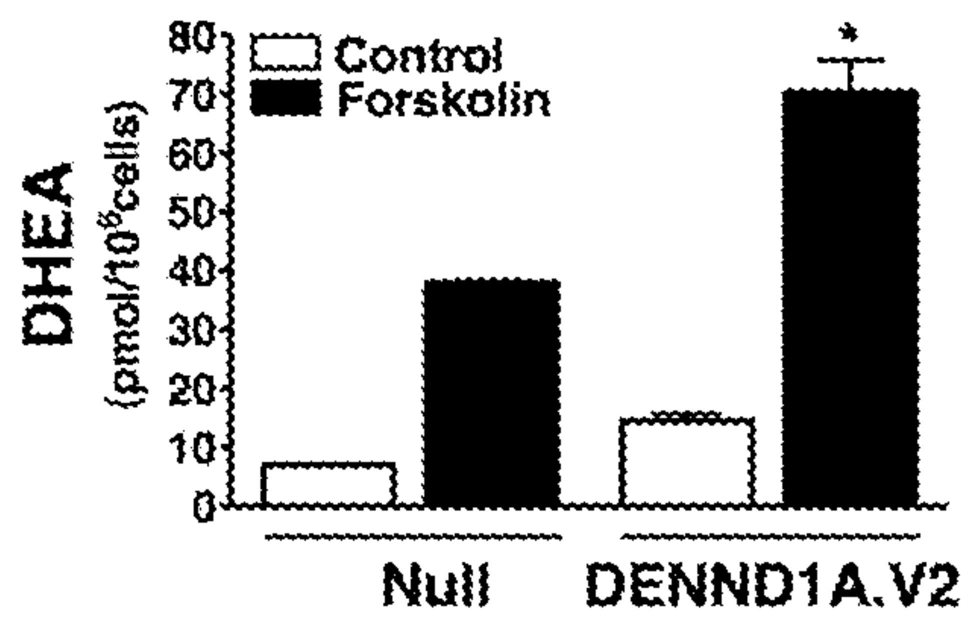


Figure 5B

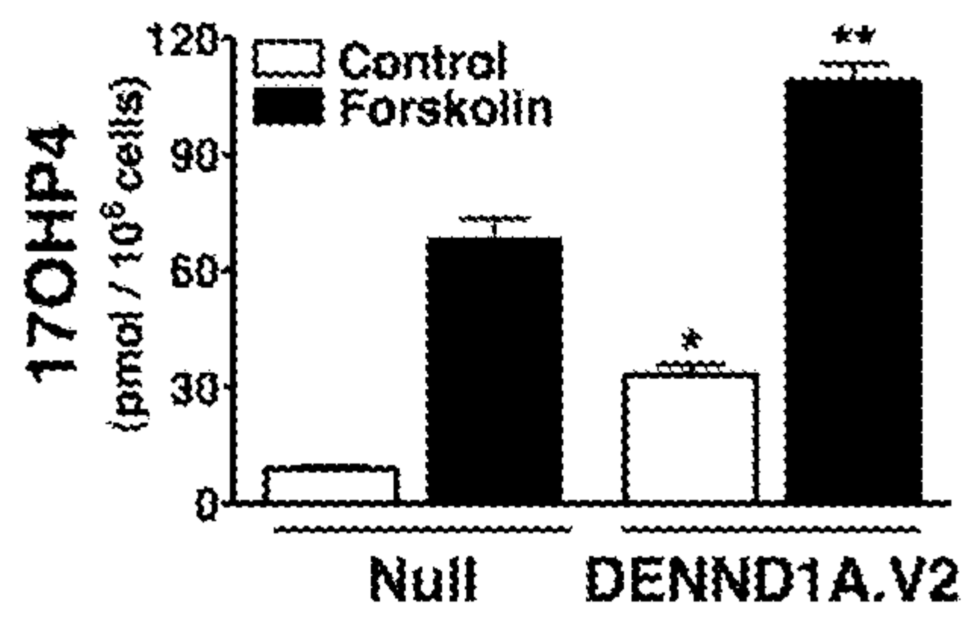


Figure 5C

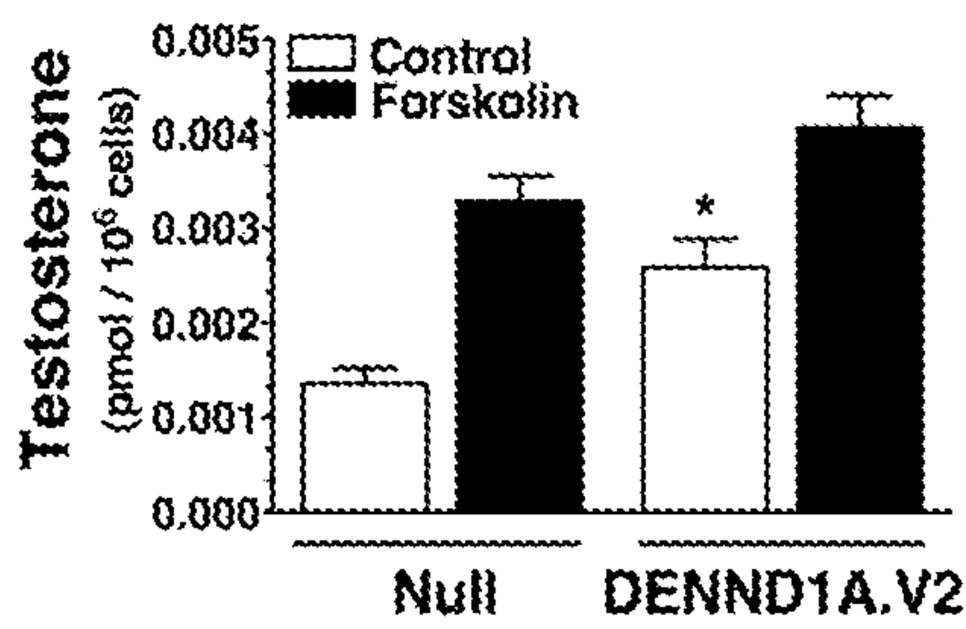
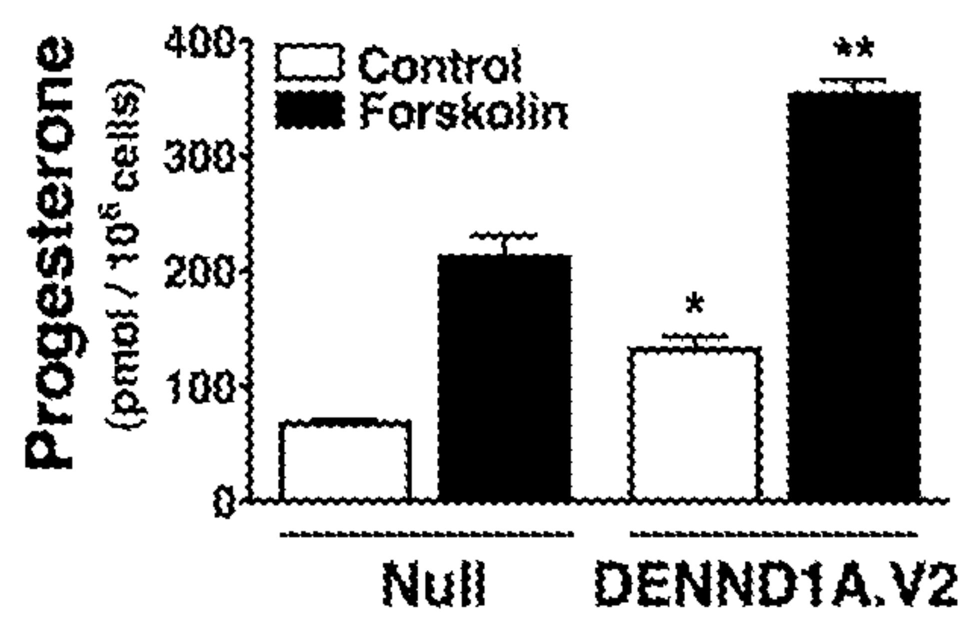


Figure 5D



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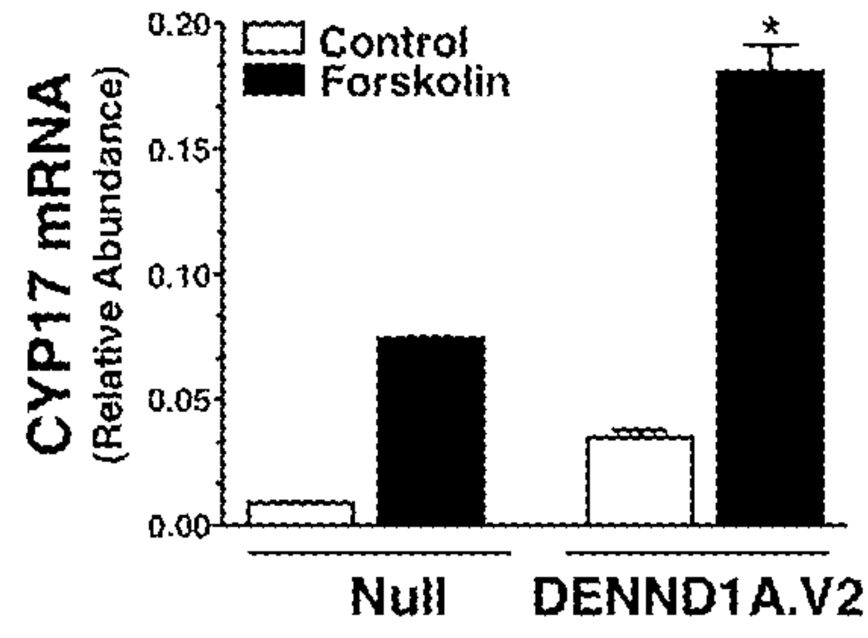


Figure 6A

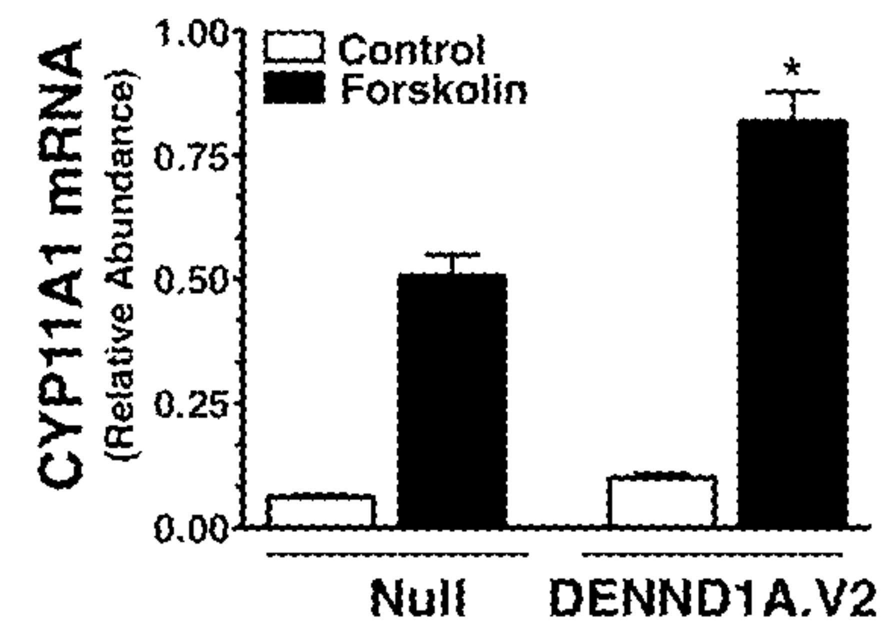


Figure 6B

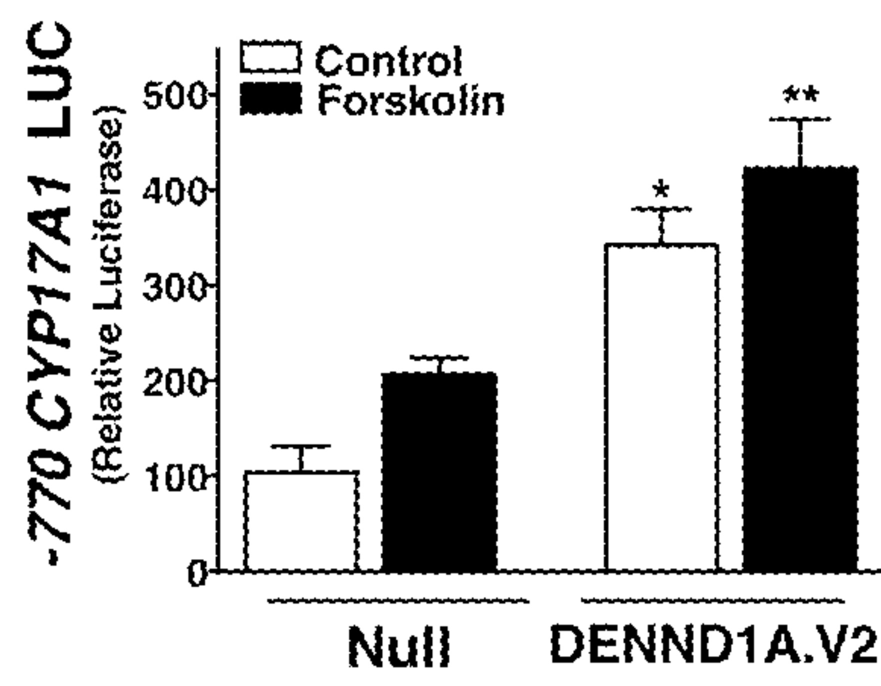


Figure 6C

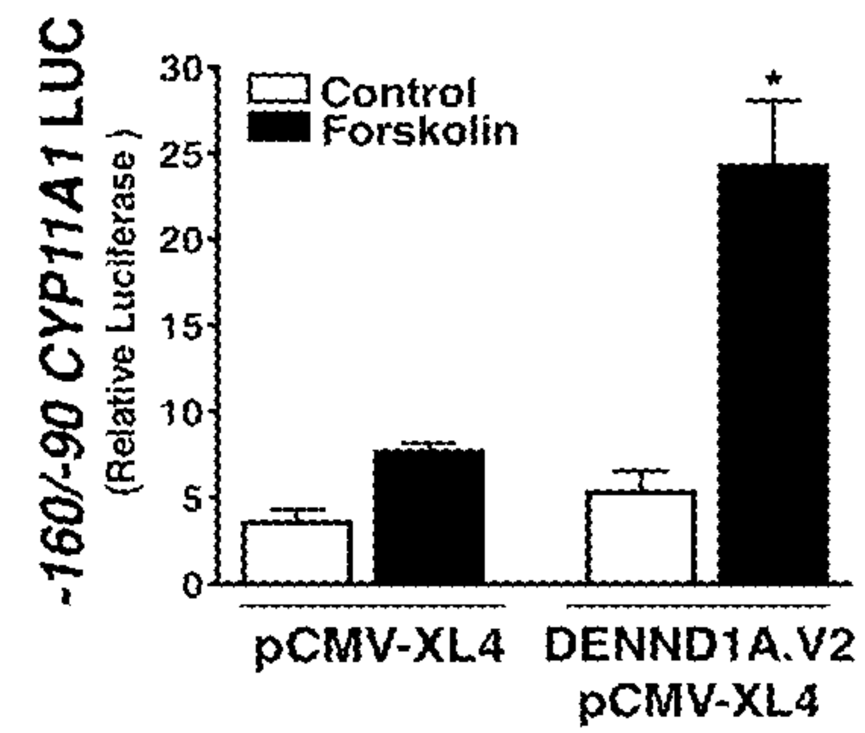


Figure 6D

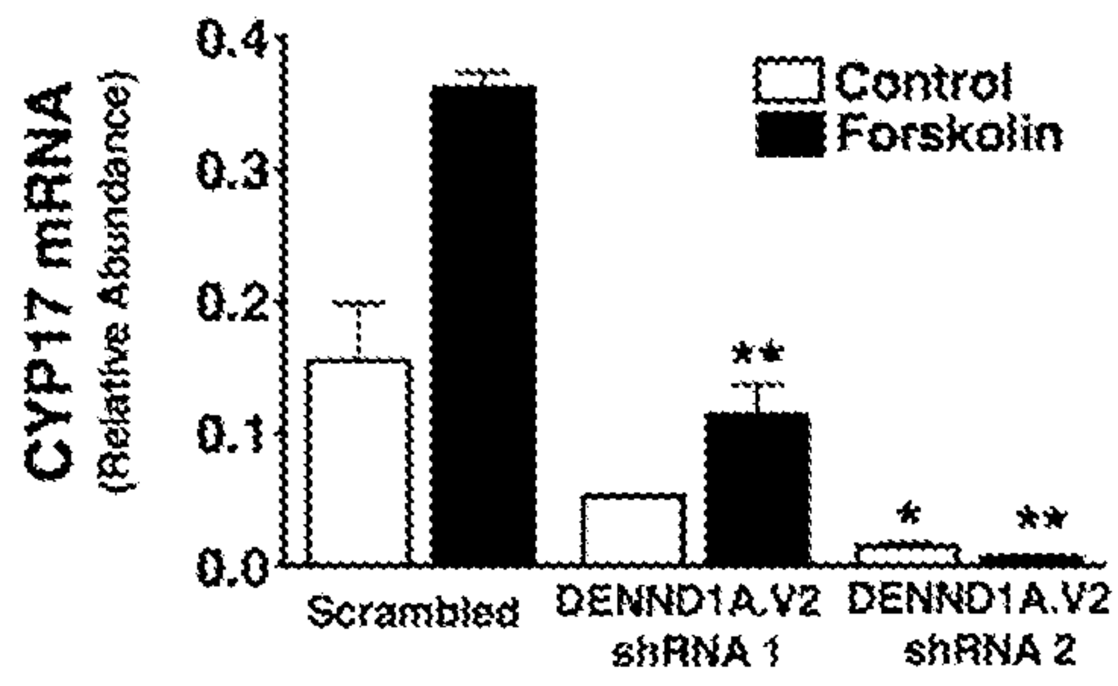
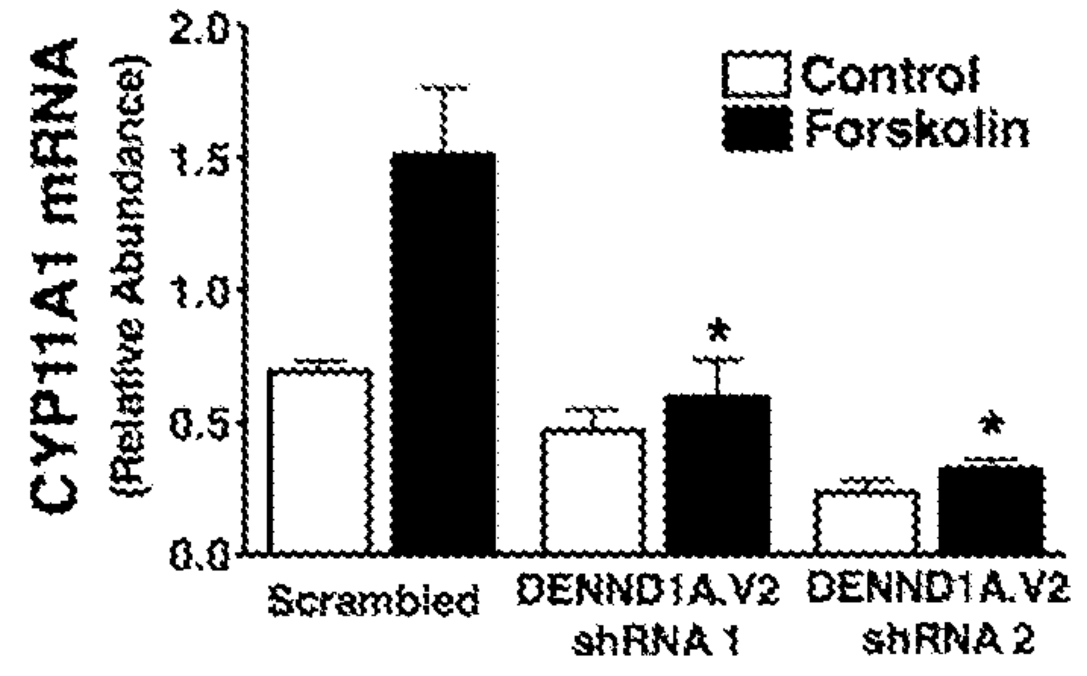


Figure 7A



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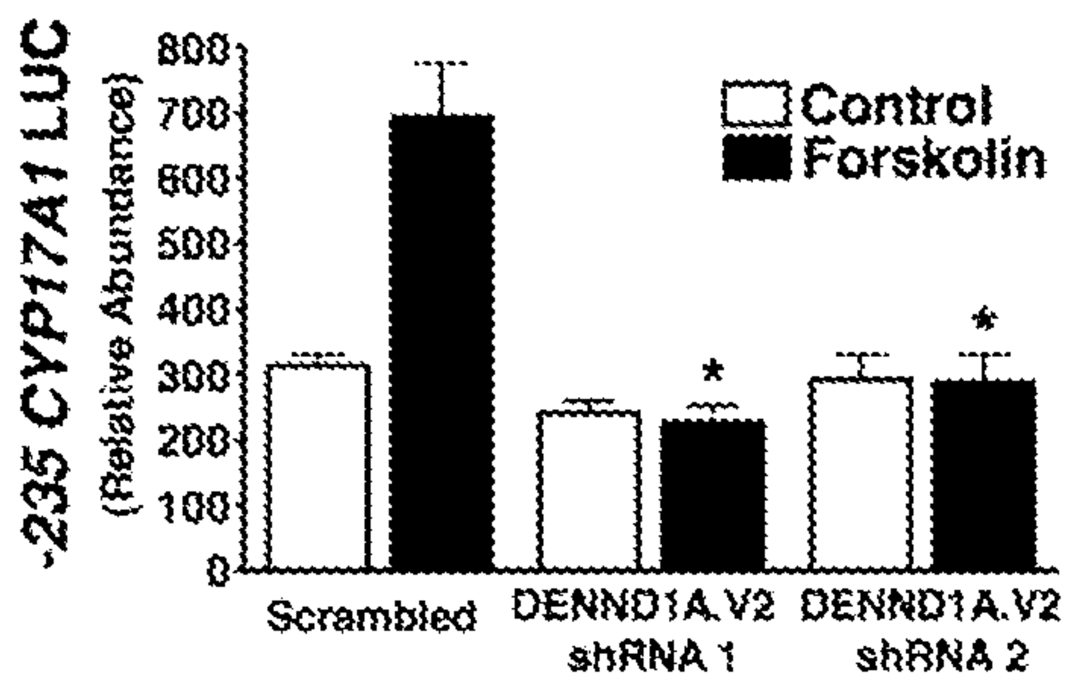

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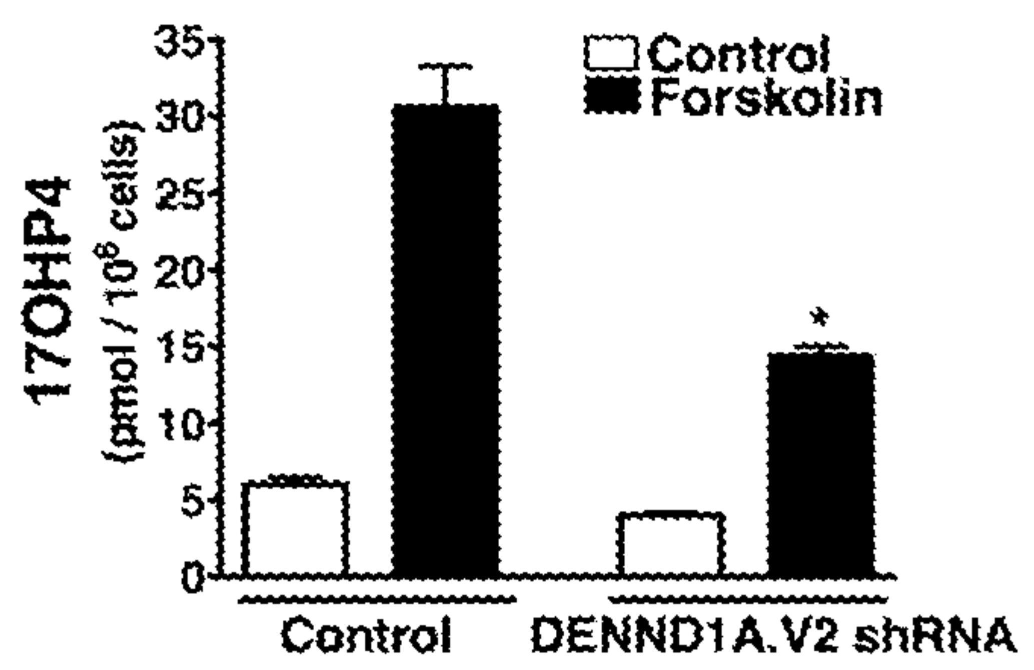

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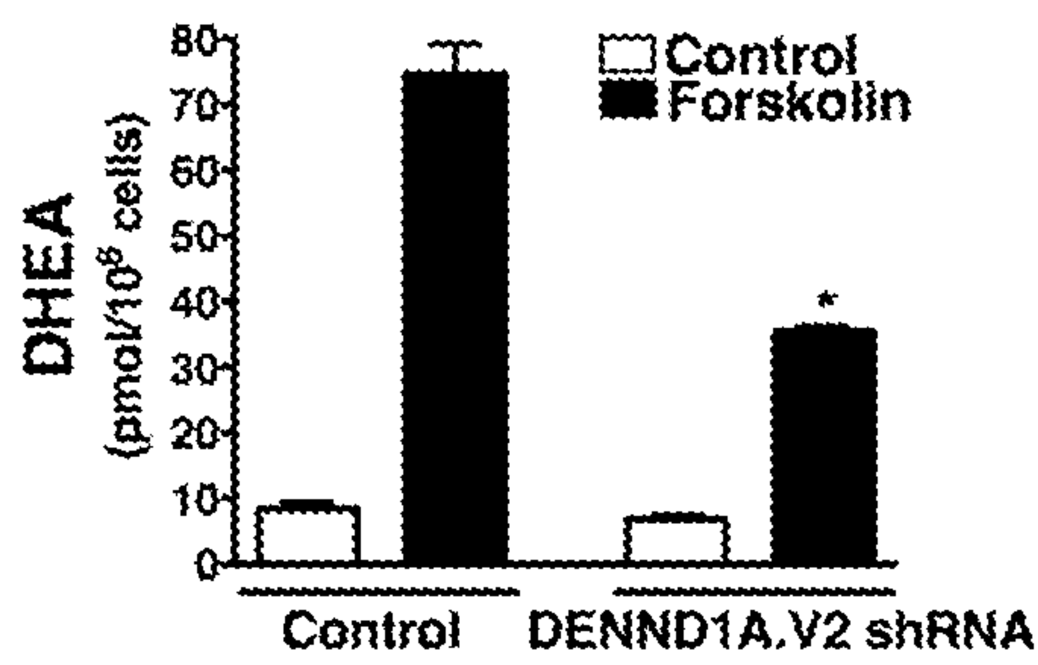

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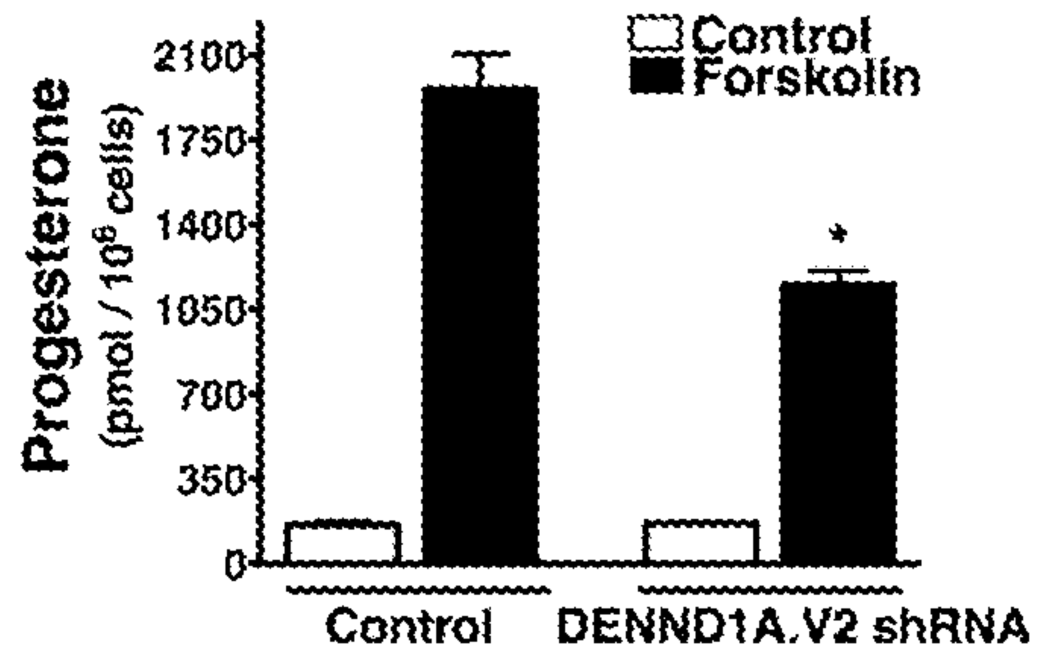


Figure 7F

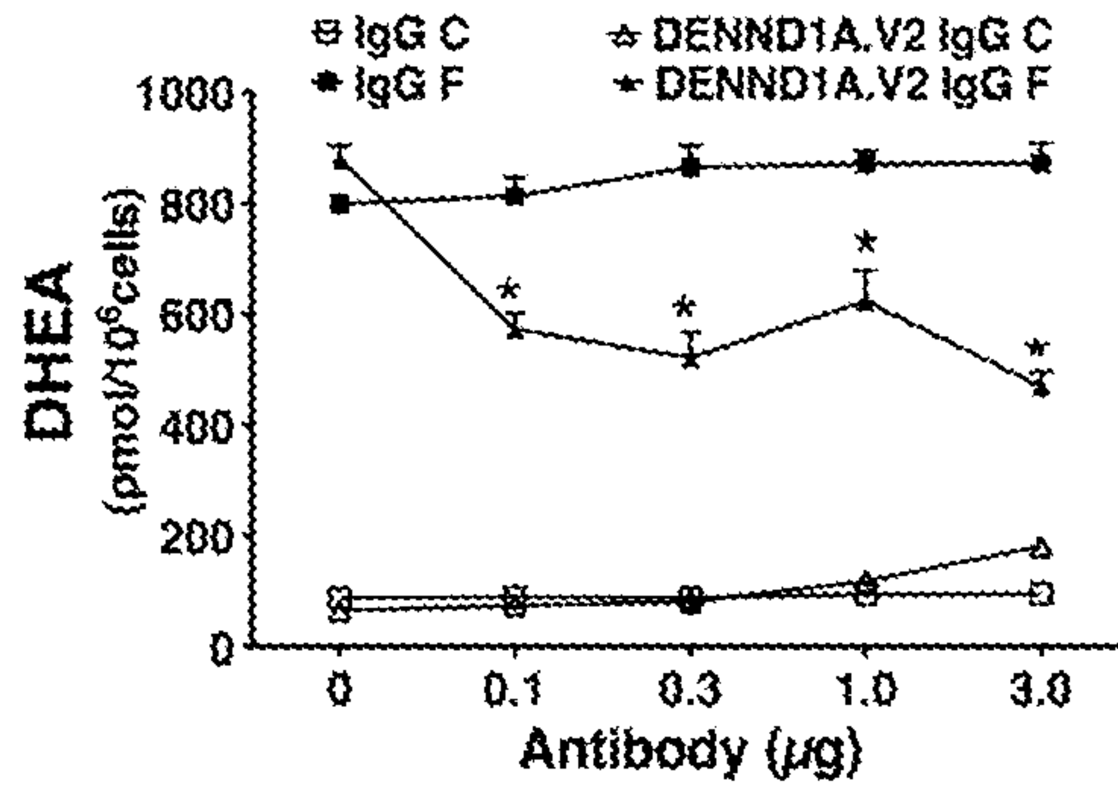
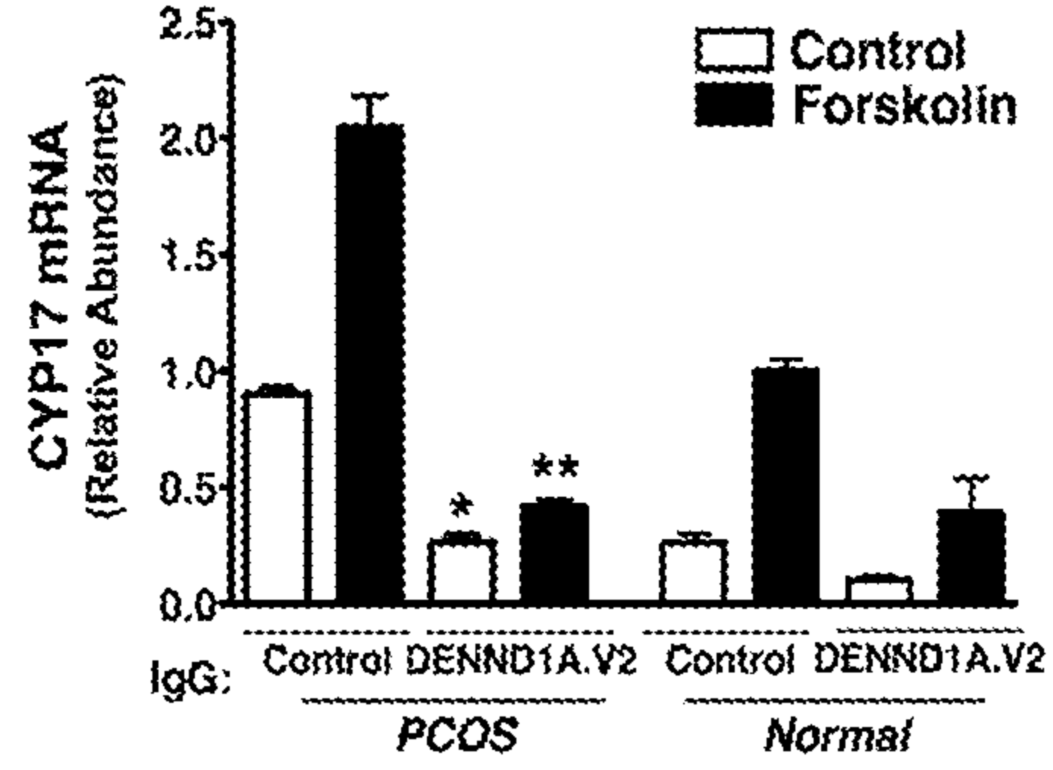


Figure 8A



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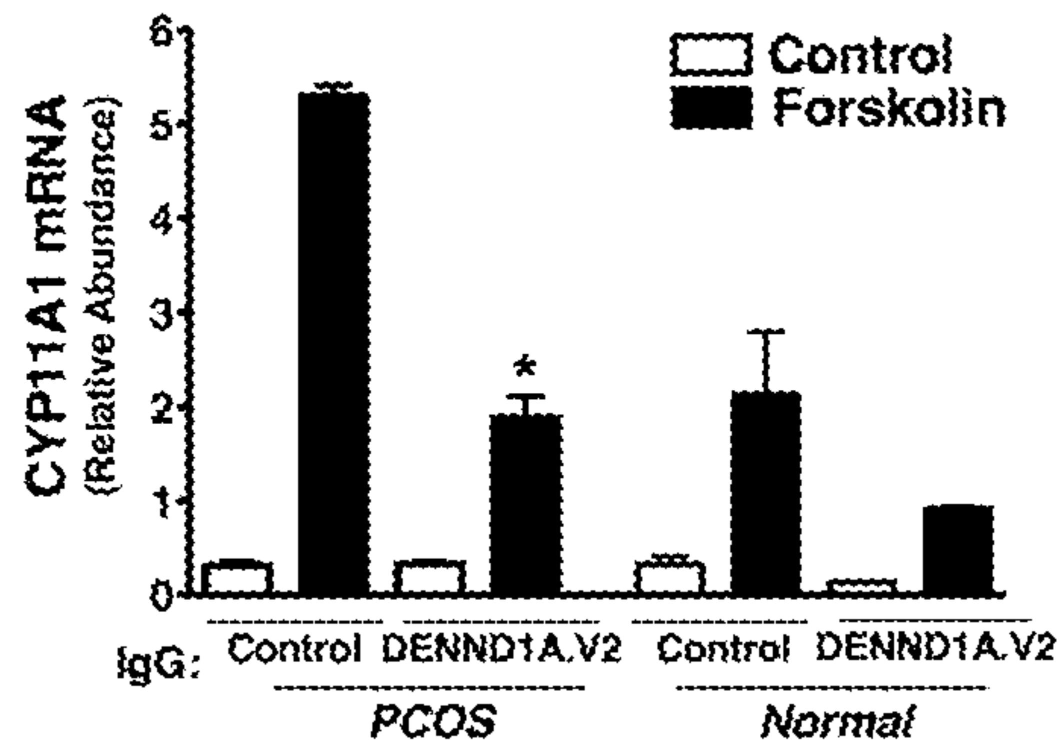

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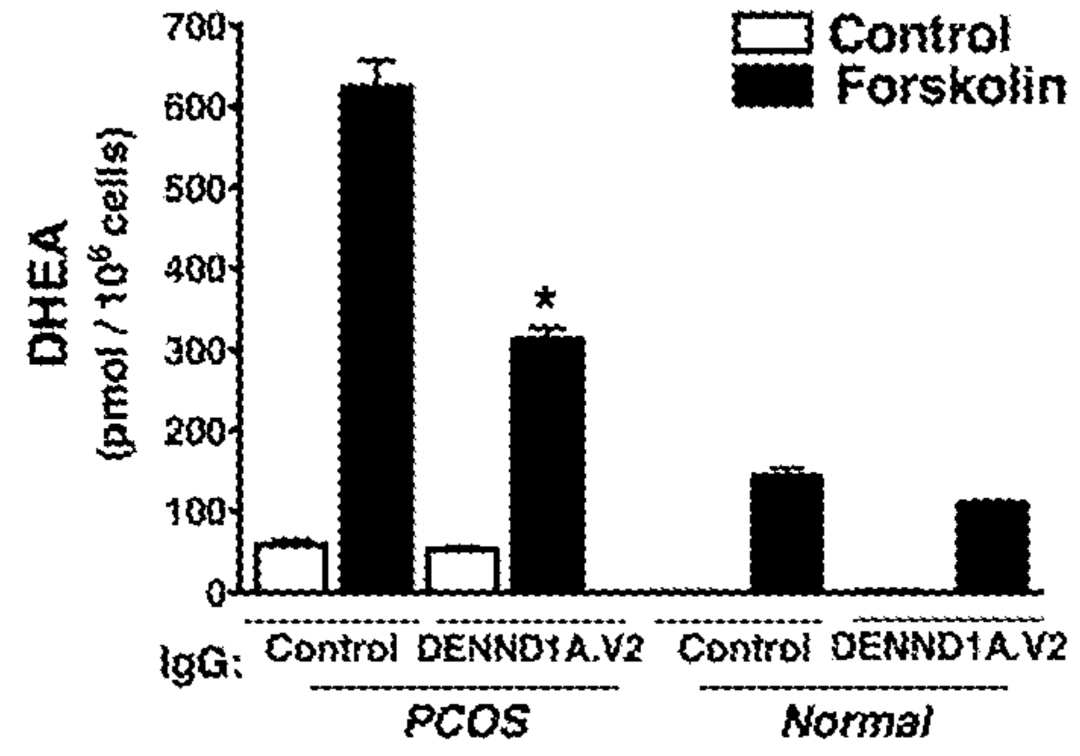

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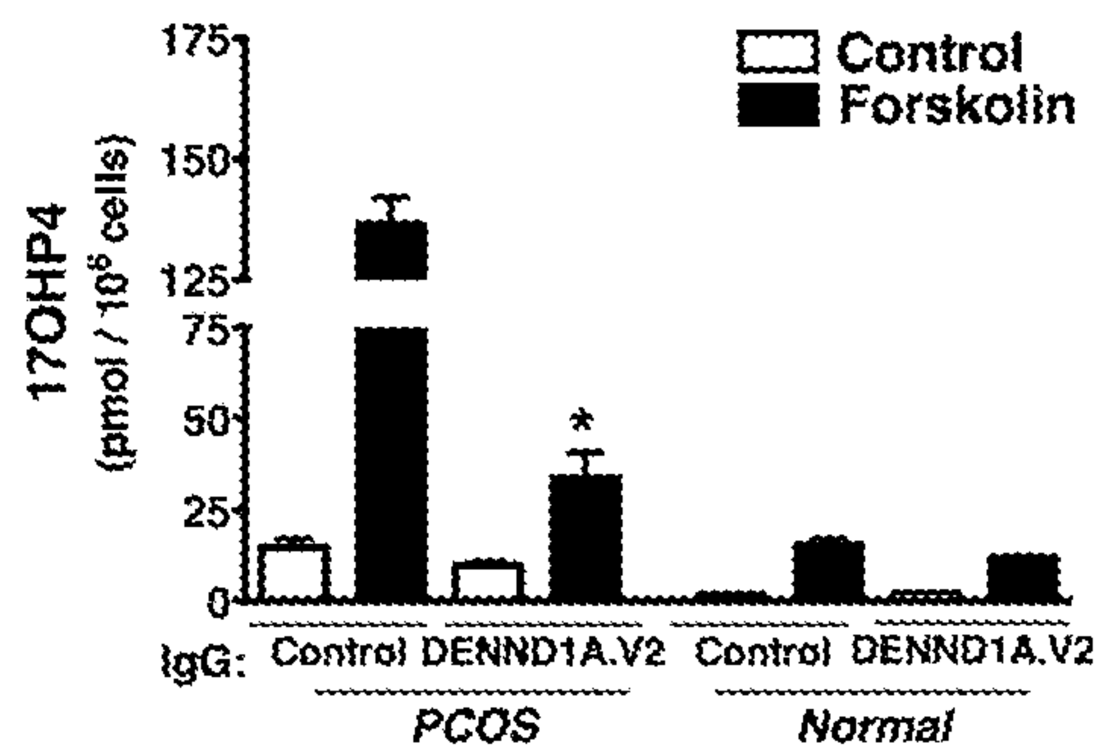

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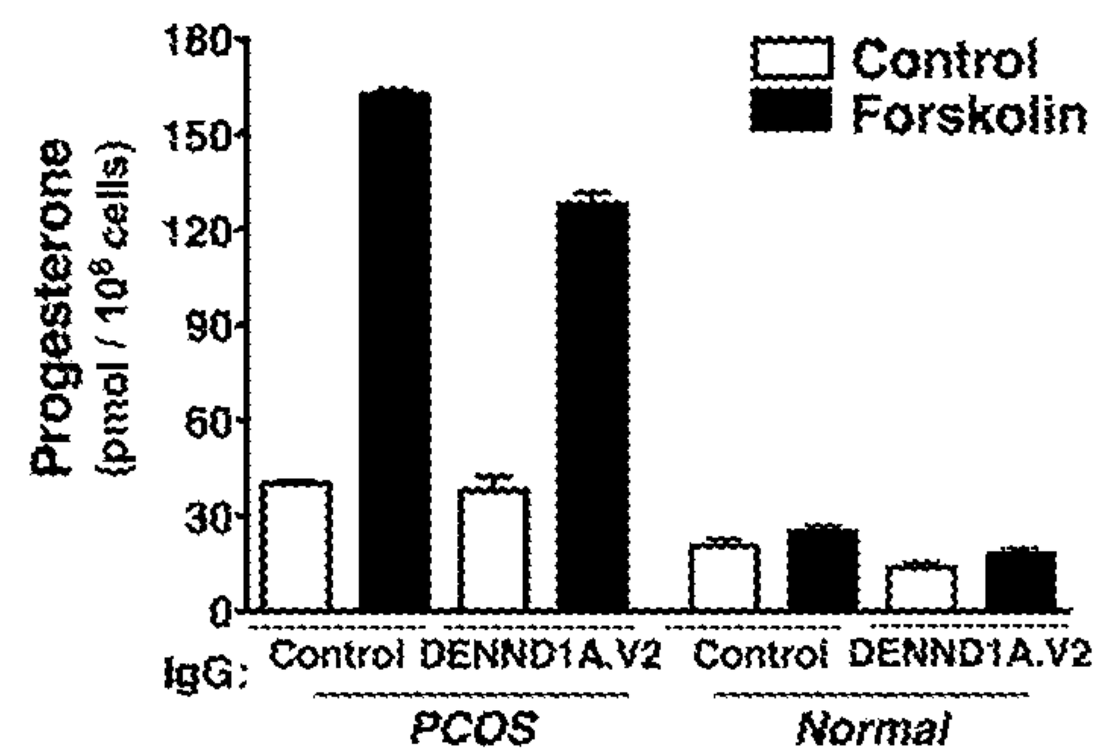


Figure 8F

scFV DNA

VH-linker-*VL*,

**5′-GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTC
TGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAATTA
TTGGTACTGATGGTGATGATACAAATTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAG
AACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGCTGAAGCTAG
TTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGC**ggtggaggcggttcaggcggaggtggcagcggcg
gtggcgggtcgacg***GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATC
ACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCT
GATCTATGGTGCATCCGATTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTC
TCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGTATGATTCTGCTCCTAGTACGTTC
GGCCAAGGGACCAAGGTGGAAATCAAACGG***–3′** (SEQ ID NO:52)

Figure 9A

scFv Protein

VH-linker-VL, CDRs framed in boxes

**EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSIIGTDGDDTNYADSVKGRFTISRDNSKN
TLYLQMNSLRAEDTAVYYCAKAEASFDYWGQGTLVTVSS**ggggsggggsggggs**t*DIQMTQSPSSLSASVGDRVTIT
CRASQSISSYLNWYQQKPGKAPKLLIYGASDLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYDSAPSTF
GQGTKVEIKR*** (SEQ ID NO:53)

Figure 9B

DNA Sequence of the Heavy Chain of Human Recombinant DENND1.V2 IgG1

5'_*atggatagccgtctgaacctggtcttcctggtcctgattctgaaaggggtg*__GAGGTGCAGCTGTTGGAGTCTGG__
GGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCA
TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAATTATTGGTACTGATGGTGATGATACAAAT
TACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAG
CCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGCTGAAGCTAGTTTTGACTACTGGGGCCAGGGAACCC
TGGTCACCGTCTCGAGCgctagcaccaagggcccatcggtcttccccctggcaccctcctccaagagcacctctggg
ggcacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccct
gaccagcggcgtgcacaccttcccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgc
cctccagcagcttgggcacccagacctacatctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagaaa
gttgagcccaaatcttgtgacaaaactcacacatgcccaccgtgcccagcacctgaactcctggggggaccgtcagt
cttcctcttccccccaaaacccaaggacaccctcatgatctcccggacccctgaggtcacatgcgtggtggtggacg
tgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccg
cgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaa
ggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagc
cccgagaaccacaggtgtacaccctgcccccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctg
gtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccac
gcctcccgtgctggactccgacggctccttcttcctctatagcaagctcaccgtggacaagagcaggtggcagcagg
ggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccg
ggtaaa_**3'**(SEQ ID NO:56)

Figure 10A

DNA Sequence of the Light Chain of Human Recombinant DENND1.V2 IgG1

5'_*atgggttggtcctgtattatcctgttcctggtcgctactgctactggggtgcatagt*___**GACATCCAGATGACCCA**___
___**GTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCT**___
___**ATTTAAATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGGTGCATCCGATTTGCAAAGTGGG**___
___**GTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTT**___
___**TGCAACTTACTACTGTCAACAGTATGATTCTGCTCCTAGTACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGT**___a
cggtggctgcaccatctgtcttcatcttcccgccatctgatgagcagttgaaatctggaactgcctctgttgtgtgc
ctgctgaataacttctatcccagagaggccaaagtacagtggaaggtggataacgccctccaatcgggtaactccca
ggagagtgtcacagagcaagacagcaaggacagcacctacagcctcagcagcaccctgacgctgagcaaagcagact
acgagaaacacaaagtctacgcctgcgaagtcacccatcagggcctgagctcgcccgtcacaaagagcttcaacagg
ggagagtgt_**3'**(SEQ ID NO:57)

Figure 10B

Protein Sequence of the Heavy Chain of Human Recombinant DENND1.V2 IgG1

VH- [CDRs underlined and bracketed]
*MDSRLNLVFLVLILKGVQC*__EVQLLESGGGLVQPGGSLRLSCAASGFTFS[SYAMS]WVRQAPGKGLEWVS[IIGTDG__
__DDTNYADSVKGRFT]ISRDNSKNTLYLQMNSLRAEDTAVYYCAK[AEASFDY]WGQGTLVTVSSA__STKGPSVFPLAP
SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS
NTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ
KSLSLSPGK (SEQ ID NO:58)

Figure 10C

Protein Sequence of the Light Chain of Recombinant DENND1.V2 IgG1

VL- [CDRs are underlined and bracketed]
*MRPSIQFLGLLLFWLHGAQC*___DIQMTQSPSSLSASVGDRVTITC[RASQSISSYLN]WYQQKPGKAPKLLIY[GASDL___
___QS]GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC[QQYDSAPSTF]GQGTKVEIKR___TVAAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPV
TKSFNRGEC (SEQ ID NO:59)

Figure 10D

Project ID: Phage Display Screening
DENND1A.V2 IgG1

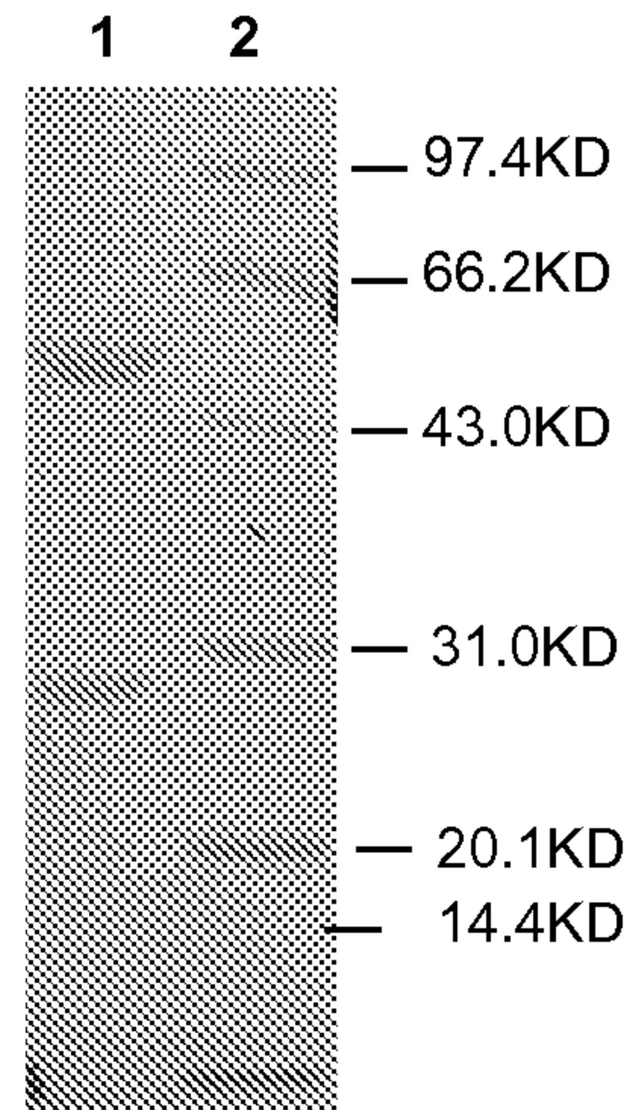
Product Type: IgG1

Label ID	Volume	Concentration	Total Amount
79	1.0 mL	0.126 mg/mL	1.0 mg

Method of Production: A plasmid vector was transfected into 293E cells. The suspension culture was collected 96 hours after transfection. The product was purified by HiTrap rProteinA FF and filtered by 0.2 μm.
Concentration Measurement: BCA method
Calculated Concentration 126 μg/mL
Total Amount: 1008 μg Formulation Buffer: 0.1M glycine-HCl, 0.05M Tris, pH7.4

Figure 11A

Reducing SDS-PAGE



Lane1: DENND1A.V2 IgG1
Lane2: Marker

Figure 11B

ELISA result

Items	OD490
79	1.079
Medium	0.076
PBS	0.052

Coating: Bio-CH-22
2nd Ab: HRP-goat anti human IgG1

Figure 11C

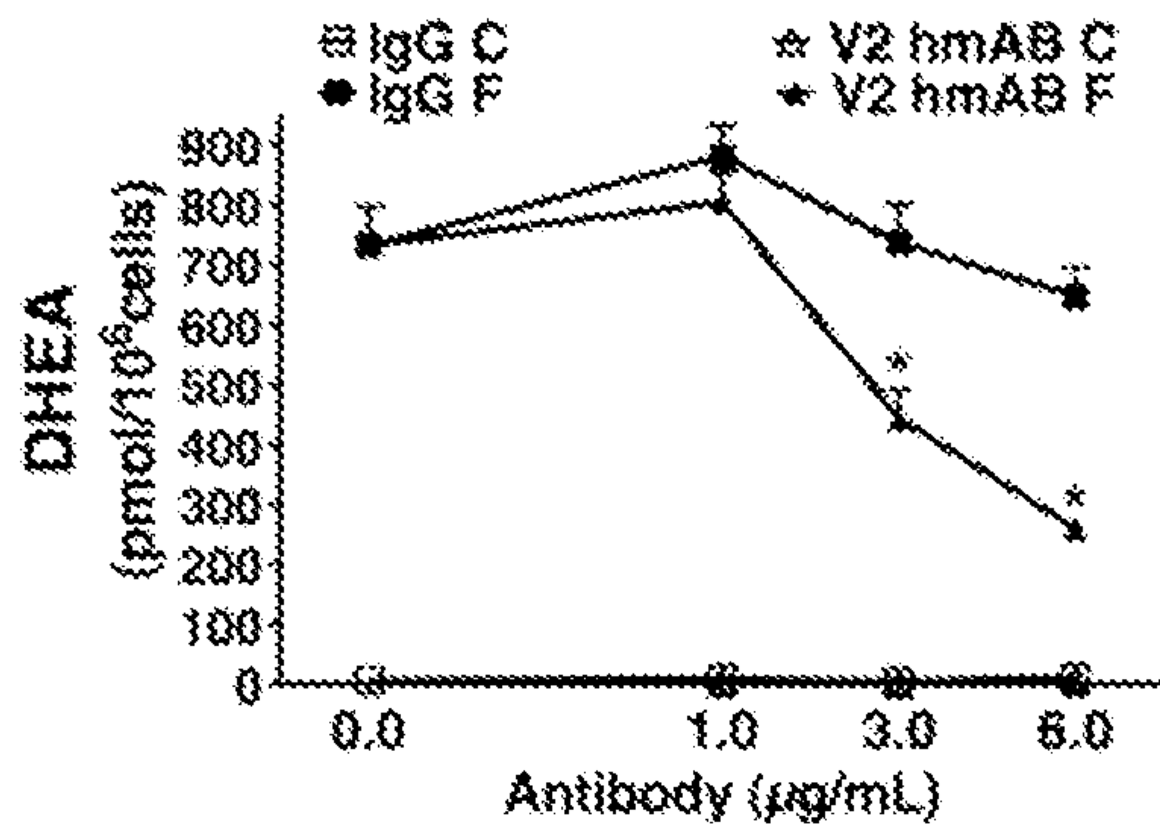


Figure 12A

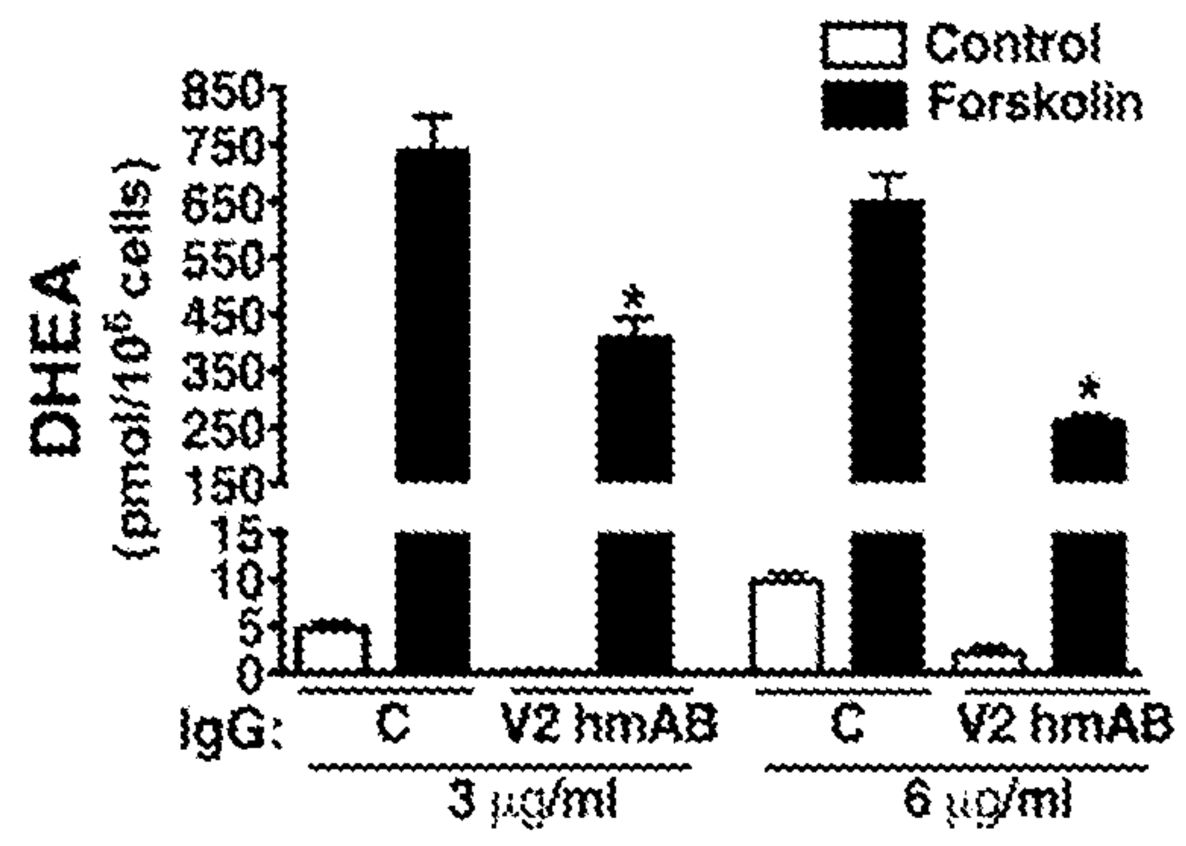


Figure 12B

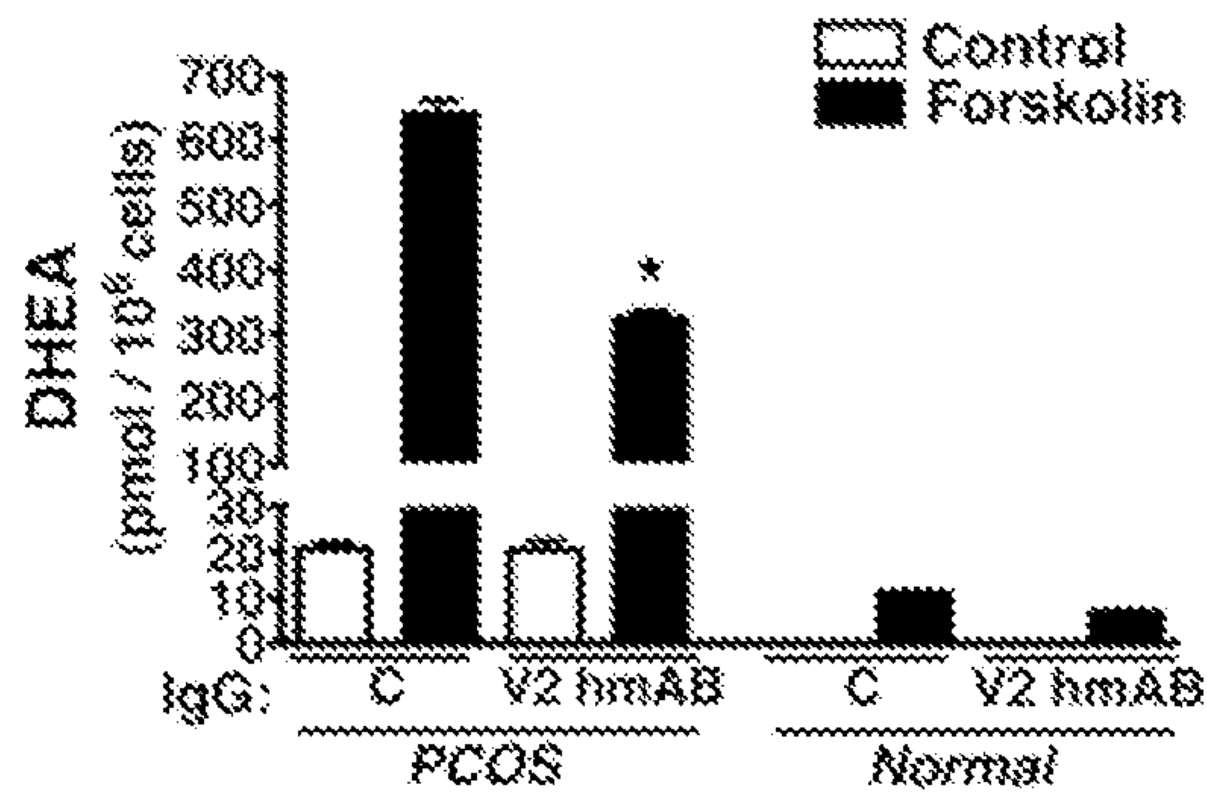


Figure 13A

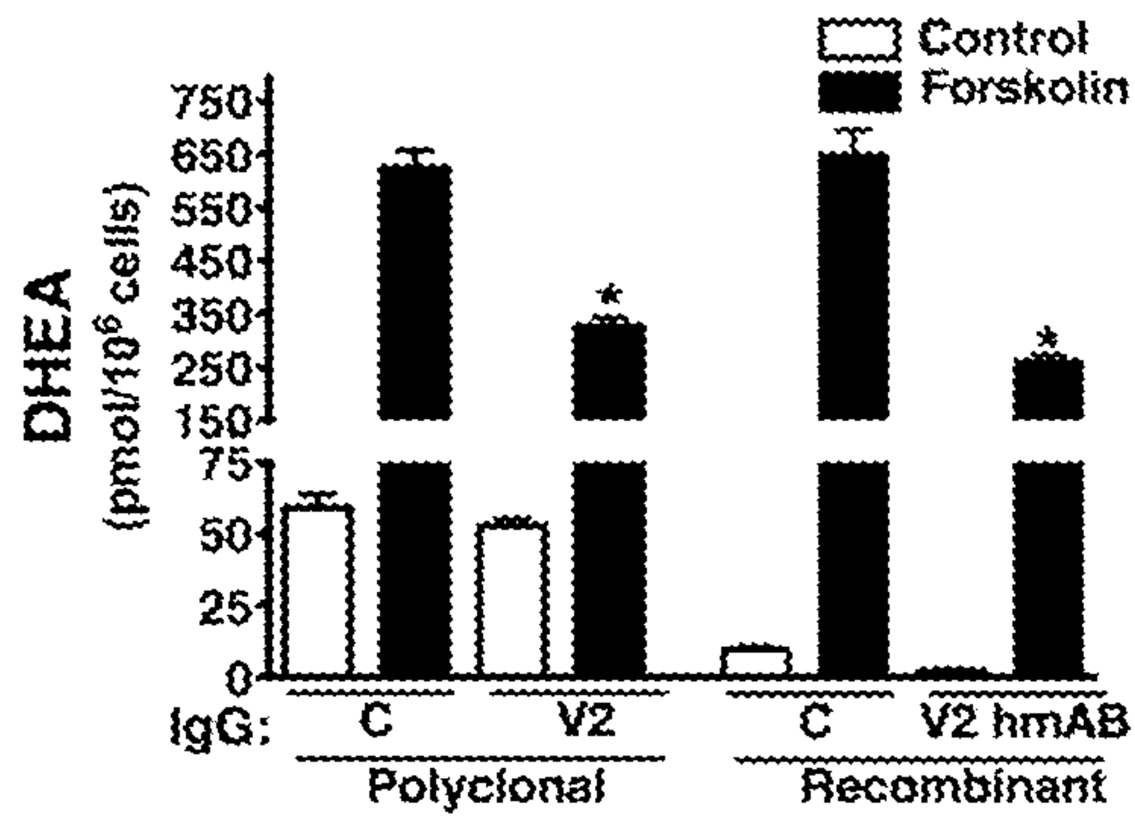


Figure 13B

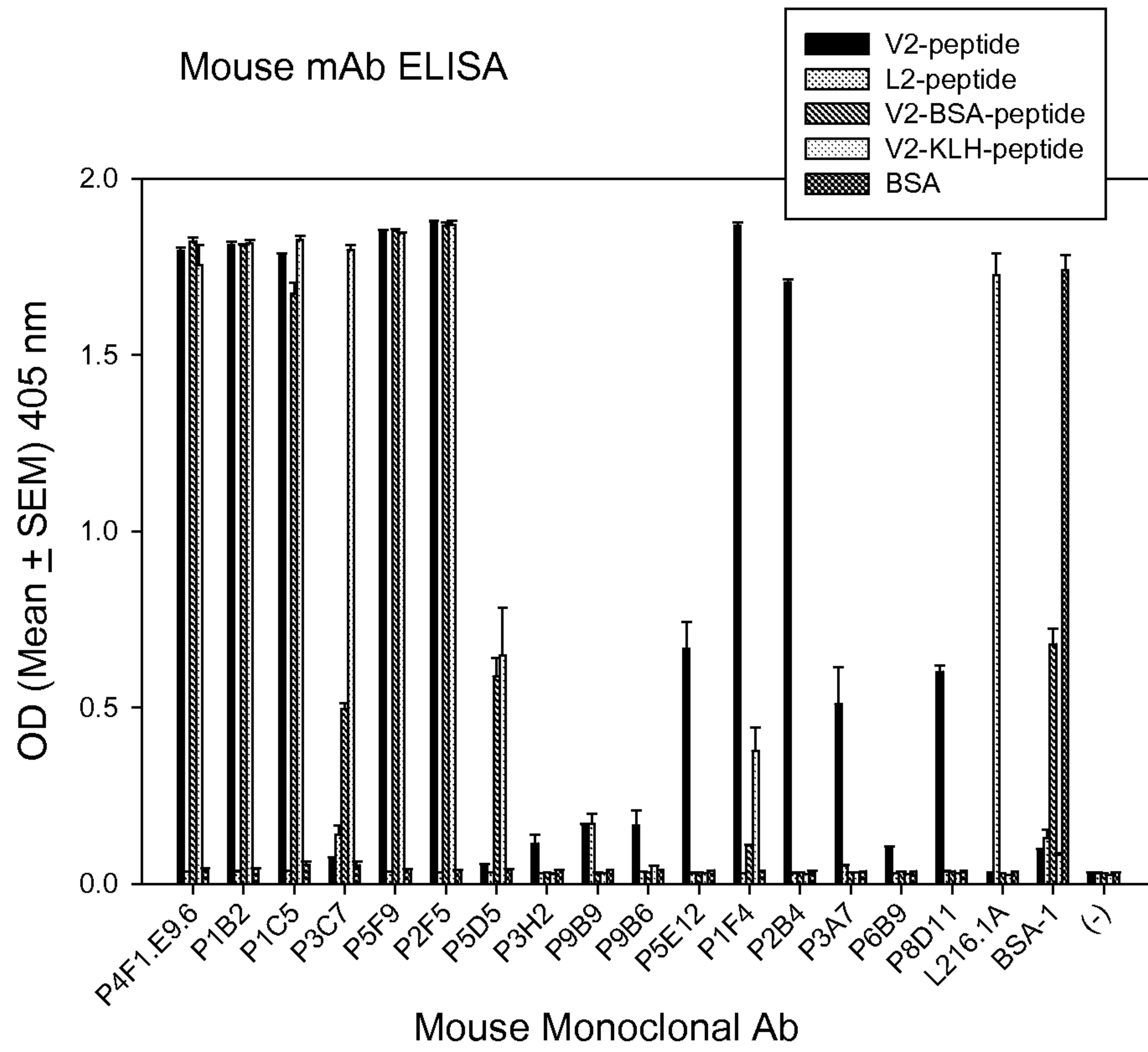


Figure 14

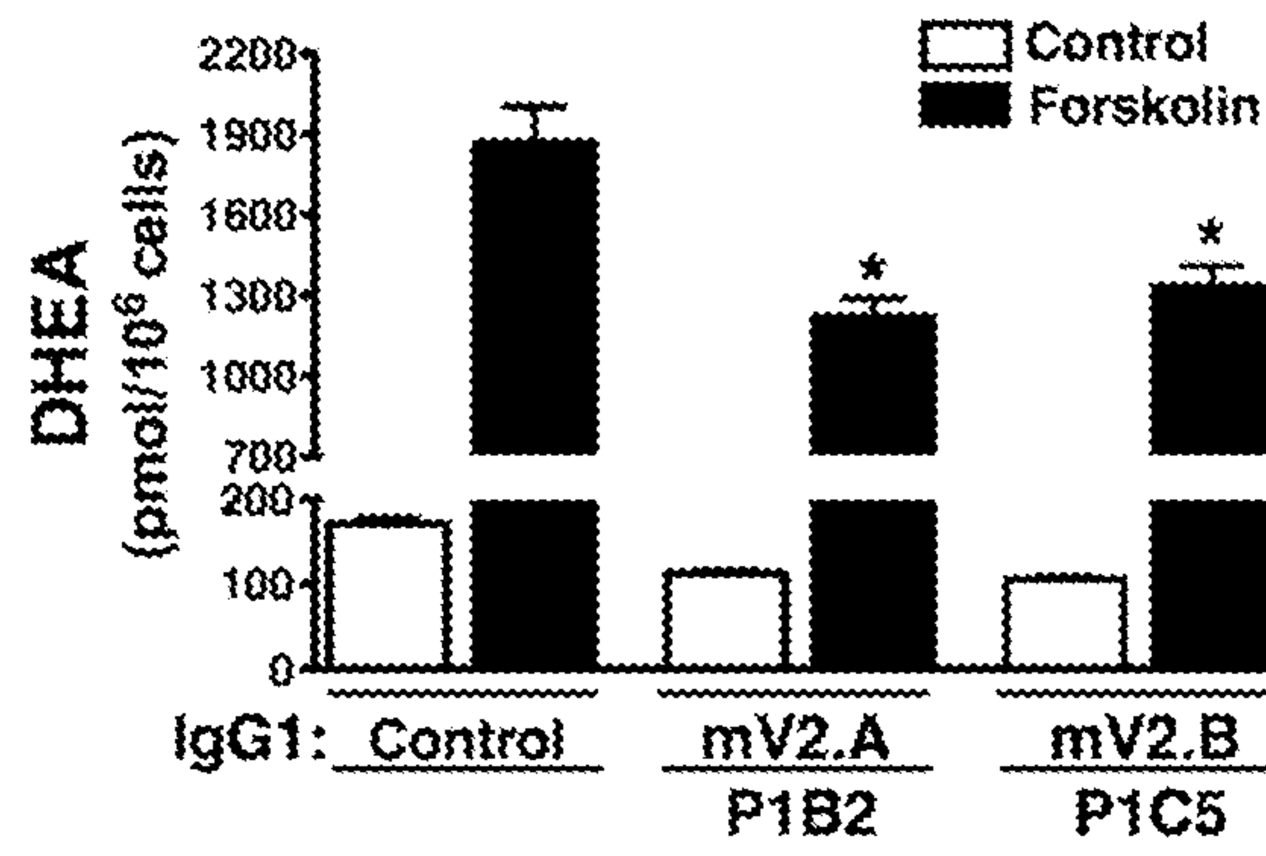


Figure 15

P1B2 mouse mAB DNA Heavy Chain sequence

**CAGCAGTCTGGGGCAGACCTTGTGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCTGGCTTCAACAT
TAAAGACTTCTATATGCACTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAGTGGATTGGATGGATTGATCCTG
AGAATGGTGATACTGATTATGCCCCGAAGTTCCAGGGCAGGGCCACTATGACTGCAGACACATCCTCCAACACA
GCCTACCTGCAGCTCAACAGCCTCACATCTGAGGACACTGCCGTCTATTACTGTAATGCCCATACCTTCCTGCA
TGGTAACTCCGAACCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCC** (SEQ ID NO:60)

Figure 16A

P1B2 mouse mAB DNA Light Chain sequence

***ATTGTGATGACCCAGTCTCCAAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACCTGCAAGGCCAG
TCAGGATGTGATTGCTGCTGTTGCCTGGTATCAACAGAAACCAGGACAATCTCCTGAACTACTGATTTACTCGG
CATCCTACCGCTACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGGACGGATTTCACTTTCACCATC
AGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGTCAGCAACATTATAGTACTCCGTGGACGTTCGGTGG
AGGCACCAAGCTGGACATCAAACGGGCT*** (SEQ ID NO:61)

Figure 16B

P1B2 mouse mAB Heavy Chain Protein Sequence

VH- [CDRs are underlined and bracketed]

*QQS***GADLVRSGASVKLSCTAS[GFNIKDFYMH]WVKQRPEQGLEWIG[WIDPENGDTD]YAPKFQGRATMTADT
SSNTAYLQLNSLTSEDTAVYYCNA[HTFLHGNSEPMDY]WGQGTSVTVS** (SEQ ID NO:62)

Figure 16C

P1B2 mouse mAB Light Chain Protein Sequence

VL- [CDRs are underlined and bracketed]

*IVMTQSPKFMS****TSVGDRVSITC[KASQDVIAAVA]WYQQKPGQSPELLIY[SASYRYT]GVPDRFTGSGSGTDF
TFTISSVQAEDLAVYYC[QQHYSTPWT]FGGGTKLDIKRA*** (SEQ ID NO:63)

Figure 16D

P1C5 mouse mAB DNA Heavy Chain sequence

**CAAGTNCAGCTGCAGCAGTCTGGGGCAGAGCTTGTGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTC
TGGCTTCAACATTAAAGACTACTATATGCACTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAGTGGATTGGAT
GGATTGATCCTGAGAATGGTGATACTGAATATGCCCCGAAGTTCCAGGGCAAGGCCACTCTGACTGCAGACACA
TCCTCCAACACAGCCTACCTGCAACTCAGCAGCCTGACATCTGAGGACACTGCCGTCTATTACTGTAATGCCCA
CTACGGTACTAGCCAGGGGGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCC** (SEQ ID NO:64)

Figure 16E

P1C5 mAB Heavy Chain Protein Sequence

VH- [CDRs are underlined and bracketed]

**QVQLQQSGAELVRSGASVKLSCTAS[GFNIKDYYMH]WVKQRPEQGLE[WIGWIDPENGDTE]YAPKFQG
KATLTADTSSNTAYLQLSSLTSEDTAVYYC[NAHYGTSQGAMDY]WGQGTSVTVS** (SEQ ID NO:65)

Figure 16F