

Wnt-4 Protects Thymic Epithelial Cells Against Dexamethasone-Induced Senescence

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Abstract

Glucocorticoids are widely used immunosuppressive drugs in treatment of autoimmune diseases and hematological malignancies. Glucocorticoids are particularly effective immune suppressants, because they induce rapid peripheral T cell and thymocyte apoptosis resulting in impaired T cell-dependent immune responses. Although glucocorticoids can induce apoptotic cell death directly in developing thymocytes, how exogenous glucocorticoids affect the thymic epithelial network that provides the microenvironment for T cell development is still largely unknown. In the present work, we show that primary thymic epithelial cells (TECs) express glucocorticoid receptors and that high-dosage dexamethasone induces degeneration of the thymic epithelium within 24 h of treatment. Changes in organ morphology are accompanied by a decrease in the TEC transcription factor FoxN1 and its regulator Wnt-4 parallel with upregulation of lamina-associated polypeptide 2 α and peroxisome proliferator activator receptor γ , two characteristic molecular markers for adipose thymic involution. Overexpression of Wnt-4, however, can prevent upregulation of adipose differentiation-related aging markers, suggesting an important role of Wnt-4 in thymic senescence.

Introduction

AUTOIMMUNE DISEASES AND HEMATOLOGICAL malignancies are significant causes of morbidity and mortality world wide.^{1,2} Although research is ongoing, treatment options are still often limited to high-dosage synthetic glucocorticoid (GC) analogs despite their nonspecificity and multiple side effects. Indeed, GCs are still applied in therapy for acute and chronic autoimmune diseases and hematological malignancies,^{3,4} because they effectively promote apoptosis of leukemia cells⁵ and trigger complex anti-inflammatory actions by influencing both molecular and cellular components of the immune system.⁶ Apart from triggering decreased expression of cytokines and major histocompatibility complex class II (MHC II), GCs also induce apoptotic death of peripheral⁷ and developing T cells. In mouse models, GCs cause massive thymocyte depletion, especially in the CD4⁺CD8⁺ (double positive [DP]) thymocyte population,^{8–12} blocking *de novo* T cell production.

Prior experiments have also demonstrated that high-dose GCs induce a dramatic¹³ and apoptosis-associated¹⁴ involution of the thymus, and not only thymocytes but also thymic epithelial cells (TECs) are seriously affected.¹⁵ Additionally, a recent report by Fletcher et al.¹⁶ has highlighted that TEC

depletion appears reversible, and thymic epithelial stem cells play an important role in this process.

Because physiological steroids are implicated in the regulation of aging,^{17,18} we theorized that GC treatment might affect thymic epithelial senescence. Although morphological similarities between physiological and induced thymic involution are striking, to date the process has not been studied in detail at the molecular level. One possible mechanism is that during physiological aging TECs undergo epithelial-to-mesenchymal transition (EMT) and then proadipose differentiation.^{19,20} Our studies have recently provided evidence that this process is regulated by Wnt-4 and FoxN1 decline, leading to drastic reduction in TEC identity^{21,22} and simultaneous upregulation of lamina-associated polypeptide (LAP) 2 α as well as preadipocyte-related markers peroxisome proliferator activator receptor (PPAR) γ and adipose differentiation-related protein (ADRP).²⁰

On the basis of the above studies, we theorized that GCs do not simply deplete thymocytes and the majority of TECs, but they also inhibit the function of the remaining epithelium via downregulation of characteristic TEC markers, leading to preadipocyte differentiation. In the present study, we provide evidence that both primary TECs and the primary TEC-derived TEPI cell line express glucocorticoid receptors (GRs)

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and respond to GC treatment. Upon a single high-dose GC injection, gene expression levels of both Wnt-4 and FoxN1²¹ become significantly downregulated, resulting in upregulation of preadipocyte differentiation markers (LAP2 α , PPAR γ). Regeneration of TECs—including normal FoxN1 level—occurs within 3 months after dexamethasone (DX) injection. However, if GC-treatment is continued with repeated small doses as in clinical applications, then Wnt-4 and FoxN1 transcription cannot recover and aging markers remain locked at elevated levels. Overexpression of Wnt-4 in the FoxN1-deprived TEP1 cell line is, however, able to protect against GC-induced adipose transdifferentiation, indicating Wnt-4 as the primary protector of thymic epithelium against adipose involution.

Materials and Methods

Mice and treatment of animals

Four-week-old BALB/c mice were used for the experiments. Animals received a single-dose (20 mg/kg) DX (Oradexon, Organon) injection intraperitoneally (i.p.) in phosphate-buffered saline (PBS) and then were sacrificed 24 and 168 h after injection; control animals received PBS. Another group of mice received PBS and DX for 3 months, respectively. There was also a group of mice receiving one high dose of DX, then a continuously low dose of DX (2 mg/kg) on every second day for a month, to mimic the therapeutic regimen of autoimmune diseases.^{23,24} All animal experiments were carried out in accordance with the regulations set out by Pécs University's committee on animal experimentation (#BA 02/2000-2/2006).

Cell lines and in vitro DX treatment

The TEP1 cell line was maintained and used for experiments as described.²⁰ The Wnt-4-overexpressing TEP1 cell line was generated as described previously.²⁰ Cell lines were treated with DX (Sigma, dissolved in dimethylsulfoxide [DMSO] until use) with a final concentration of 1 μ M for 1 week or solvent, respectively.

Preparation of TECs

Thymic lobes were digested with 3 mg/mL collagenase II (GIBCO) for 30 min, then washed with Dulbecco modified Eagle medium (DMEM) 10% fetal calf serum (FCS). Cell suspensions were then labeled with anti-EpCAM1-FITC (clone G8.8) and washed with magnetic cell sorting (MACS) buffer followed by incubation with anti-fluorescein isothiocyanate (FITC) microbeads (Miltenyi Biotec), the EpCAM1⁺-cells were used for total RNA isolation and subsequent quantitative polymerase chain reaction (PCR) analysis. The cells were purified using MACS LS separation columns (Miltenyi Biotec).

Histology using fluorescent antibodies

Frozen thymic sections (7–10 μ m thick) were fixed in cold acetone, then dried and blocked using 5% bovine serum albumin (BSA) in PBS for 20 min before staining with a-Ly51-PE (clone 6C3) and a-EpCAM1 (clone G8.8) antibodies (either indirectly coupled with a-rat Northern Light 637 secondary antibody or directly labeled with FITC). GR, ER-TR7, and

Wnt-4 protein levels were detected using an a-GR-FITC mouse monoclonal antibody (clone 5E4B1 developed in our laboratory²⁵ and commercially available at Serotec), a rat monoclonal a-ER-TR7, and a polyclonal goat a-Wnt4 antibody (Abcam). Visualization was performed using an a-rat-Ig-PE secondary antibody and a-goat Ig Northern Lights 557 (RnD systems), respectively. Parallel with GR detection, corresponding sections were incubated with an irrelevant antibody. To detect GR expression in TECs, the Olympus Fluoview 300 confocal microscope with an Olympus Fluoview FV1000S-IX81 system was used. All the other sections were analysed using an Olympus BX61 microscope equipped with CCD camera and AnalySIS software.

RNA isolation, preparation of cDNA

Total RNA was isolated using RNeasy plus kit (Qiagen), following the manufacturer's instructions. Following RNA isolation, DNase digestion was performed using a DNase I digestion kit (Sigma) and cDNA was reverse transcribed using a high-capacity RNA to cDNA kit according to the manufacturer's instructions (Applied Biosystems).

Quantitative real-time and qualitative reverse transcriptase PCR analysis of purified TECs and cell lines

For real-time PCR analysis, we used an ABI 7500 Software system and ABI SYBR Green PCR master mix. The expression levels of LAP2 α 2, PPAR γ , ADRP, and Wnt-4 were analyzed and normalized to the level of 18S rRNA. The qualitative expression of GR and CD45 in TEC was verified by PCR using ReddyMix (ABgene) according to the manufacturer's instructions, with 18S rRNA as an internal control. The PCR products were visualized on agarose gels. The sequences of primers are listed in Table 1. In the case of FoxN1 and AIRE, TaqMan chemistry was used for PCR reaction and analysis, and the levels of these genes were normalized to the TaqMan HPRT1 expression. PCR reactions were run for a maximum of 40 cycles.

Statistical analysis

Data are presented as mean \pm standard deviation (SD), and the effects between various experimental groups were compared with the Student t-test. $p < 0.05$ was considered as significant.

Results

GR expression in primary thymic epithelium and the TEP1 cell line

GCs regulate cellular function via GR, which belongs to the nuclear receptor superfamily²⁶ and is required for the regulation of development and homeostasis of various epithelial-like tissues.^{27,28} Using reverse transcriptase (RT)-PCR analysis (Fig. 1A) and confocal laser scanning microscopy (Fig. 1B), it was demonstrated that apart from thymocytes^{8,9} GR is also expressed in primary TECs and the TEP1 cell line, confirming their ability to respond directly to GC stimuli.

Effects of single-dose GC administration

To study how the thymic epithelial network is affected by GC, sections of DX-treated and control thymi were stained

TABLE 1. LIST OF GENE-SPECIFIC PRIMERS

Gene	Forward primer	Reverse primer
CD45	5'-CCG GAA TTC CGG ATG GGT TTG TGG CT-3'	5'-CCG CTC GAG CGG CTA ATC ACT GGG TG-3'
GR	5'-TGG TGT GCT CCG ATG A-3'	5'-AGG GTA GGG GTA AGC-3'
FoxN1	Applied Biosystems TaqMan probe PN4351272 (Mm00477457_m1)	
LAP2 α	5'-TGA ACT GCA GGC AGC TAA GA-3'	5'-TCA TAG CTA GAC TCT GAG G-3'
PPAR γ	5'-CCC AAT GGT TGC TGA TTA CAA A-3'	5'-AAT AAT AAG GTG GAG ATG CAG GTT CT-3'
ADRP	5'-CGC CAT CGG ACA CTT CCT TA-3'	5'-GTG ATG GCA GGC GAC ATC T-3'
Wnt4	5'-CTC AAA GGC CTG ATC CAG AG-3'	5'-TCA CAG CCA CAC TTC TCC AG-3'
18S rRNA	5'-GGG TCG GGA GTG GGT AAT TT-3'	5'-AGA AAC GGC TAC CAC ATC CAA-3'
HPRT1 Taqman	Applied Biosystems TaqMan probe Mm0046968_m1	
AIRE	Applied Biosystems TaqMan probe Mm00477457_m1	

GR, Glucocorticoid receptor; PPAR γ , peroxisome proliferator activator receptor; ADRP, adipose differentiation-related protein; AIRE, autoimmune regulator.

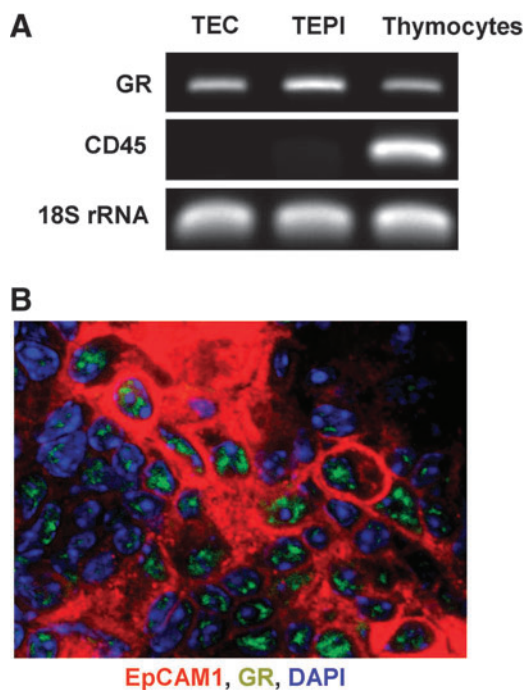


FIG. 1. Glucocorticoid receptor (GR) expression in the thymic epithelial cell (TEC) and TEPI cell lines. cDNA generated from mRNA of highly purified adult TECs and TEPI cells were tested in end point reverse transcriptase polymerase chain reaction (RT-PCR) analysis for GR expression. cDNA generated from thymocyte mRNA was used as positive control for GR expression, whereas TEC purity was tested for hematopoietic cell contamination using CD45 primers. 18S rRNA was used as an internal control (A). GR protein was detected by confocal microscopy using a-GR-FITC-labeled antibody (green) in frozen sections of untreated adult BALB/c thymi. TECs were detected using a-EpCAM1 antibody visualized by a-rat-Northern Light 637 secondary antibody (red). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) (B). Corresponding sections were probed with an irrelevant antibody as a staining control. Data shown above are representative of three separate experiments.

for the general epithelial cell marker, EpCAM1 and Ly51²⁹ to differentiate between medulla and cortex. Within 24 h, DX treatment induced marked reduction in epithelial cell-surface markers (Fig. 2B), particularly affecting the medullary (EpCAM1⁺⁺Ly51⁻) thymic compartments. The remaining TECs were purified from GC-treated thymi based on EpCAM1 expression. In the purified TEC population, gene transcription was analyzed using quantitative real-time RT-PCR. During embryonic development, TEC maturation is regulated by Wnt-4²¹; therefore Wnt-4 levels were assayed. Quantitative real-time RT-PCR analysis and immunohistochemistry confirmed that both Wnt-4 mRNA (Fig. 2A) and protein levels (Fig. 2B) decreased significantly after 24 h of DX exposure. FoxN1, a transcription factor essential for thymic organogenesis and maintenance of TEC identity directly regulated by Wnt-4 expression,³⁰ was also found to be significantly decreased (Fig. 2A). FoxN1³⁰ and Wnt-4 mRNA levels were also measured a week later and appeared to remain significantly low (Fig. 2A).

As physiological thymic involution correlates with the upregulation of preadipocyte markers LAP2 α ²⁰ and PPAR γ , the expression of these markers was also tested following DX treatment-induced involution. One day, 1 week, 1 month, and 3 months after DX exposure, TECs were purified and then analyzed using quantitative (q) RT-PCR. At day 1, the expression of LAP2 α ²⁰ and PPAR γ was low (data not shown), but it became upregulated a week later (Fig. 2C), indicating that it takes several days of Wnt-4 and FoxN1 depletion to affect preadipocyte differentiation markers. By 1 month and even by 3 months after DX treatment, Wnt-4 mRNA expression only partially increased while FoxN1, LAP2 α , and PPAR γ mRNA levels returned close to normal levels (within standard deviation) supporting the time frame of recovery (Fig. 3A).

To analyze how thymic morphology is affected, thymic sections were stained for EpCAM1/Ly51. Three months after DX treatment, no significant differences were detected between DX-treated and age-matched controls (Fig. 3B), except for the medulla, which still appeared somewhat shrunken in DX-exposed animals (Fig. 3B). To determine thymic medullary mass, qPCR was performed for the medullary marker autoimmune regulator (AIRE) gene.³¹ No significant difference

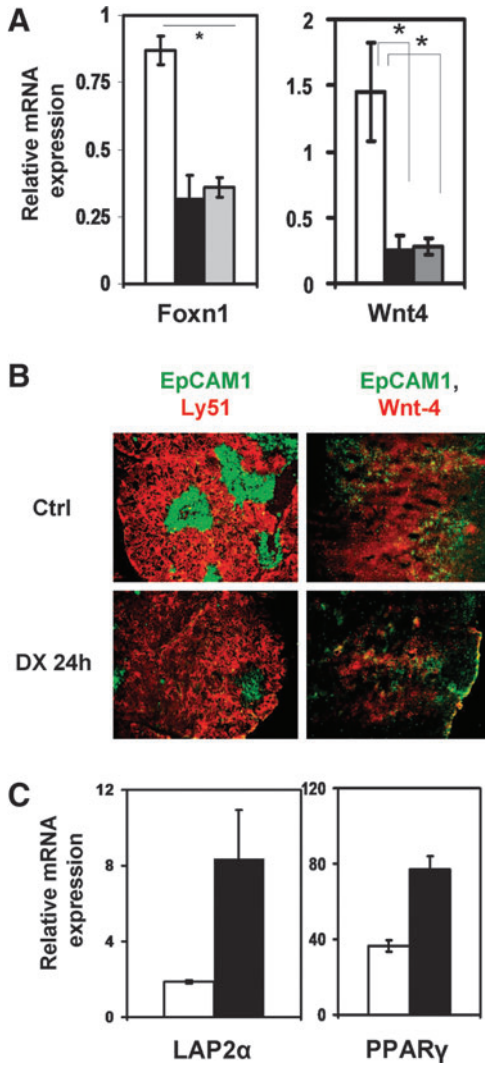


FIG. 2. Dexamethasone (DX)-induced effect on Wnt-4, Foxn1, adipocyte-related genes, and general morphology of the thymus. Foxn1 and Wnt-4 gene expression pattern (A) of purified control and DX-treated thymic epithelial cell (TEC) control (□), for 24 h (■). Gray bars (▨) represent gene expression 168 h after DX treatment and asterisks ($*p < 0.05$) indicate significant differences ($n = 3$ in each group). Note the scale differences for gene expression. Thymic sections of phosphate-buffered saline (PBS)- and DX-treated mice (24 h) were stained with a-EpCAM1-FITC (green) and a-Ly51-PE (red) monoclonal antibodies to reveal medullary and cortical compartments. The staining revealed depletion of mTECs following 24 h of DX administration. Images are representative of three independent experiments (B, left). Wnt-4 expression of control and DX-treated thymi is also shown (B, right). Wnt-4-Northern Lights 557 (red) and EpCAM1-FITC (green) in Ctrl and DX-treated thymi are presented (24 h). Images are representative of three independent experiments. Induction of adipose tissue-related genes in TEC following DX-treatment. (C) Expression of lamina associated polypeptide-2α (LAP2α), peroxisome proliferator activator receptor-γ (PPARγ), and adipose differentiation-related protein (ADRP) were tested in TECs 168 h after a single DX injection and was found to be elevated in DX-treated samples (■) (with an exception of ADRP), compared to control (□). Gene expression was normalized to 18S rRNA. All bars show means ± standard deviation (SD). Results are representative of three independent experiments ($n = 3$).

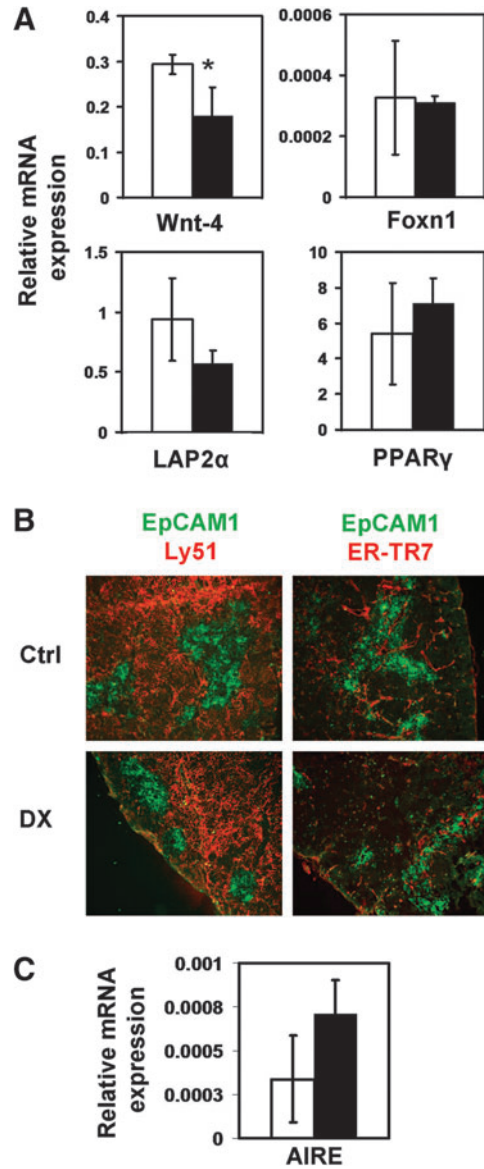


FIG. 3. Recovery of the thymic epithelial cell (TEC) compartment after dexamethasone (DX) treatment takes 3 months. (A) Bars show the gene expression changes of purified TEC: Control (□) and dexamethasone (DX) treated (■). The level of Wnt-4 remained significantly low even after 3 months ($*p < 0.05$), the levels of lamina associated polypeptide-2α (LAP2α) and peroxisome proliferator activator receptor-γ (PPARγ) were found to be unaltered, whereas adipose differentiation-related protein (ADRP) expression was found to be decreased. Note the scale differences for gene expression. Morphological analysis of age-matched control (Ctrl) versus DX-treated thymi after 3 months using EpCAM1-Ly51 (TEC network) (B, left) and EpCAM1-ER-TR7 (TEC and fibroblast) (B, right). In B (left), in the treated samples, the size of medulla appears to be smaller, but the TEC network is normal. In B (right), no remarkable differences are observed in general morphology using the ER-TR7 fibroblast marker staining on thymic sections of control and DX-treated mice. Images are representative of three independent experiments. In C, autoimmune regulator (AIRE) expression is shown in control (□) and DX- (■) purified TEC after 3 months, with no significant difference, indicating full mTEC recovery. All bars show means ± standard deviation (SD) ($n = 3$).

was detected in AIRE expression after 3 months of DX treatment (Fig. 3C), indicating the full competence of the mTEC compartment. Because EMT seems to precede preadipocyte transdifferentiation during physiological aging,²⁰ the staining pattern of fibroblast marker ER-TR7 was also examined following DX treatment. Apart from the scattered focal staining pattern of ER-TR7 in DX-treated thymi, no sign of significant EMT (Fig. 3B) was detected, supporting molecular data showing that 3 months is sufficient for TEC recovery.

Effects of sustained GC administration

To mimic the pattern of clinical applications of GCs, mice were injected with DX repeatedly for a time course of 1 month. TECs were examined and drastic downregulation of both Wnt-4 and Foxn1 mRNA levels was detected (Fig. 4A).

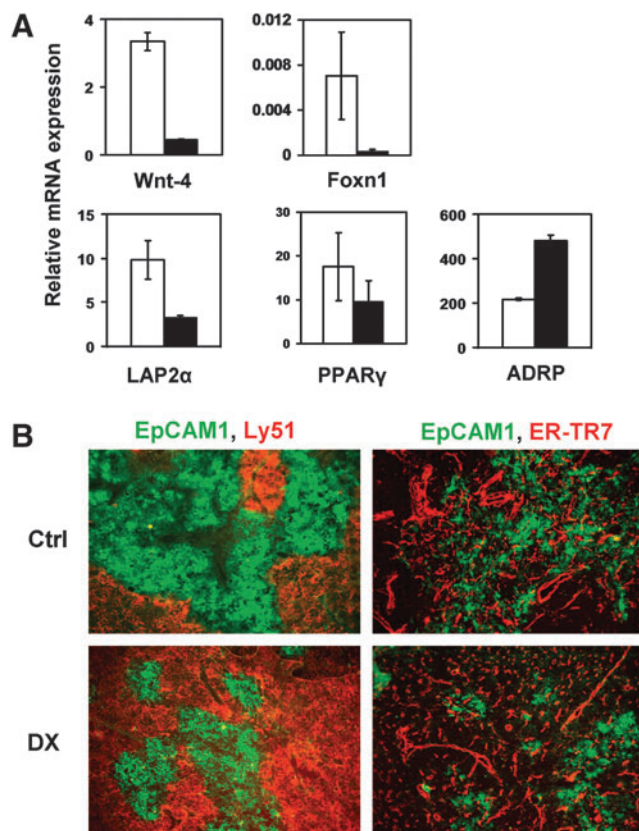


FIG. 4. Effect of continuous dexamethasone (DX) treatment on thymic epithelial cell (TEC) gene expression and architecture. (A) Bars showing the mean \pm standard deviation (SD) ($n = 3$) of Wnt-4, Foxn1, lamina associated polypeptide-2 α (LAP2 α), peroxisome proliferator activator receptor- γ (PPAR γ), and adipose differentiation-related protein (ADRP) expression of control (□) and DX-treated (■) TEC. The levels of Wnt-4, Foxn1, and LAP2 α were found to be reduced after repeated *in vivo* DX injections, whereas PPAR γ was unaltered. Additionally, ADRP was found to be elevated in DX-treated samples. Note the scale differences for gene expression. EpCAM1-Ly51 co-staining on thymus sections revealed that in DX-treated samples, the medullar area seems to be smaller compared to control (B, left). ER-TR7 staining showed no significant increase but became more punctuated in DX-treated samples. Images are representative of three independent experiments.

Although expression of early preadipocyte transdifferentiation markers LAP2 α and PPAR γ showed no significant alterations by this advanced time point, the downstream adipocyte differentiation factor ADRP was significantly increased in purified TECs of DX-treated animals (Fig. 4A), indicating that prolonged GC treatment pushed preadipocyte type transdifferentiation significantly further than a single-dose DX injection. In harmony with the molecular data, a strong decrease in the medullary compartment (Fig. 4B) was detected while the pattern of the fibroblast marker ER-TR7 (Fig. 4B) became punctuated in DX-treated thymi.

Wnt-mediated inhibition of steroid-induced adipose transdifferentiation

On the basis of our *in vivo* experiments, Wnt-4 and FoxN1 have a primary role in GC-triggered adipose involution. However, because thymocyte and TEC interactions are vitally important for the normal homeostasis of the thymus,³¹ it was important to test whether mass depletion of thymocyte populations was the trigger to Wnt-4 and consequently FoxN1 downregulation, or was direct consequence of DX exposure. To answer this question, the TEPI thymic epithelial cell line was also exposed to DX for 1 week and then molecular changes were analyzed. While Wnt-4 gene transcription was moderately downregulated LAP2 α PPAR γ and its downstream target ADRP were significantly increased (Fig. 5A), indicating that DX treatment directly induces changes leading to preadipocyte type transdifferentiation. To test the role of Wnt-4 in this process, a Wnt-4-overexpressing TEPI cell line was created and then treated with DX for a week. True to our expectations, Wnt-4 was able to inhibit preadipocyte-type fate commitment of the cell line, and no significant changes were detected in preadipocyte-type transdifferentiation markers (Fig. 5B). Interestingly, because the TEPI cell line does not express FoxN1 (unpublished observation), it has become evident that the absence of Wnt-4 alone is required and sufficient to allow preadipocyte-type transdifferentiation, and apparently FoxN1 has no significant role in the process.

Discussion

Our studies confirm that, beside T lymphocytes,^{8,9} dendritic cells,^{32,33} and TEC lines,¹⁵ primary TECs also express GR-rendering TECs directly sensitive to GCs. Following GC exposure, and similar to physiological aging, DX treatment triggered Wnt-4 and FoxN1 downregulation, leading to increased expression of preadipocyte-type differentiation markers LAP2 α and PPAR γ .^{34,20} LAP2 α and PPAR γ levels increased within a week following DX exposure, highlighting the accelerated rate of GC-induced aging compared to physiological senescence. This is in harmony with literature data, because the involvement of PPAR γ has also been confirmed in the induction of thymic involution and ectopic adipogenesis by recent studies using Rosiglitazone,³⁵ an acknowledged PPAR γ agonist.

The described increase of LAP2 α , PPAR γ , and ADRP expression is not likely to occur due to the enrichment of either mTEC or cTEC compartments. In untreated TECs, the LAP2 α expression level is similar in both epithelial subsets (data not shown), indicating no difference in sensitivity to adipocyte transdifferentiation inducing factors.

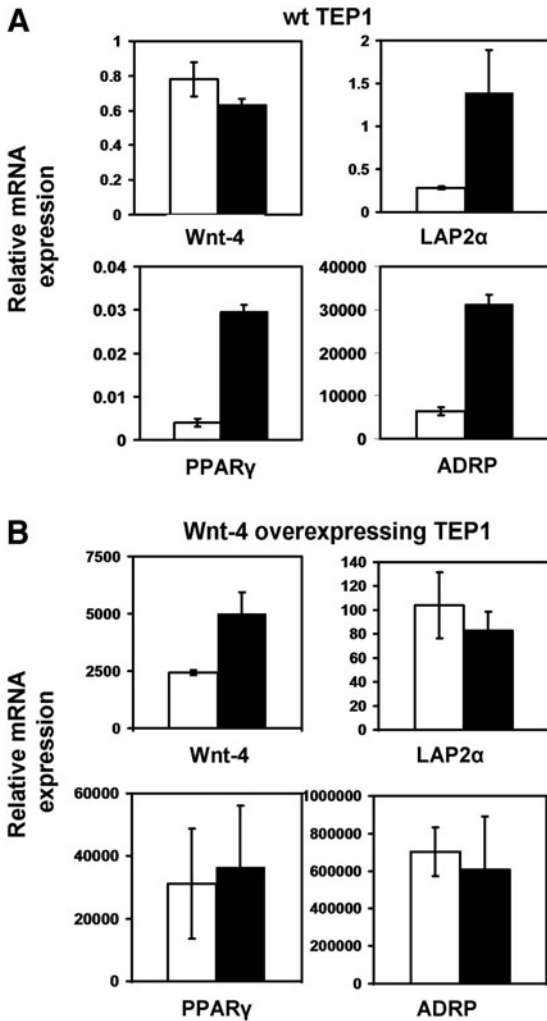


FIG. 5. Wnt-4 overexpression of the TEP1 cell line overcomes dexamethasone (DX)-induced adipocyte-related gene expression in TEP1 cells. Gene expression changes in TEP1 cell lines following 168 h of DX-treatment. The mRNA levels of Wnt-4, lamina associated polypeptide-2 α (LAP2 α), peroxisome proliferator activator receptor- γ (PPAR γ), and adipose differentiation-related protein (ADRP) was tested in solvent- and DX-treated normal (A) and Wnt-4-overexpressing TEP1 (B). White bars indicate solvent-treated control and black bars show the values of DX-treated samples. Results are representative of three independent experiments and showing means \pm standard deviation (SD). Note the different scales for gene expression.

Interestingly, as confirmed by histology, DX-treated thymi regained close-to-normal morphology 3 months following exposure, whereas DX-triggered effects were still detectable at the molecular level. Repeated DX administration resulted in drastic reduction of both Wnt-4 and FoxN1 expression and significant increase of ADRP expression, showing no sign of recovery at the mRNA level. Literature data are supportive, because prolonged decrease in FoxN1 and Wnt-4 expression have recently been demonstrated to lead to degeneration of the thymic epithelial network.³⁶ The reported phenotype was strikingly similar to age-associated involution of the thymus, except for the dramatically accelerated pace similar to that

observed following GC administration. Others reported that ubiquitous deletion of FoxN1 in the postnatal thymus has also caused thymic atrophy and severe deterioration of the TEC network.³⁷ Because FoxN1 expression is Wnt-4 dependent,²¹ Wnt-4 appears to be a realistic candidate molecule to defend the thymus against adipose involution. Using the Wnt-4-overexpressing TEP1 cell line, the working hypothesis was confirmed, because Wnt-4 overexpression could effectively block DX-induced increase of adipocyte markers and protect thymic epithelium-derived cells against DX-induced senescence at the molecular level.

In the present work, DX was shown to trigger accelerated thymic aging accompanied by thymic involution.^{13,14,17} Using markers identified in our previous studies,²⁰ we demonstrate that the processes of physiological and GC-induced accelerated thymic senescence share similar molecular mechanisms yet operate at different time scales. Because GCs are used relatively often in clinical therapies for sustained periods to suppress actual flares of autoimmune diseases, our results call attention to a currently neglected potential side effect of sustained GC treatment, namely induced accelerated thymic epithelial aging. Accelerated thymic epithelial aging impairs TEC functions, including deletion of potentially autoimmune naïve T cells, as well as allows for accumulation of T cells proliferating in the periphery. Therefore GC-induced accelerated thymic epithelial senescence could provide permissive context for the development of T cell-mediated autoimmune diseases. This would mean that GC treatment could paradoxically lead to the emergence of immune pathologies, including certain autoimmune diseases.^{38–40} Our findings concerning Wnt-mediated inhibition of GC-triggered accelerated thymic senescence are also particularly important, because Wnt-4 could potentially be a candidate molecule to inhibit adipose involution of the human thymus following GC treatment or even physiological senescence, although further *in vivo* experiments are required to clarify the potential application process.

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Author Disclosure Statement

There are no competing interests.

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